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Effects of Binge Alcohol Exposure on Canonical Wnt Signaling During Fracture Repair

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EFFECTS OF BINGE ALCOHOL EXPOSURE ON CANONICAL WNT SIGNALING DURING FRACTURE REPAIR

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
APC	adenomatous polyposis coli
BAC	blood alcohol concentration
BCA	bicinchoninic acid
BMD	bone mineral density
BMU	basic multicellular units
BRU	bone remodeling units
BSA	bovine serum albumin
Col1a1	collagen type 1, alpha 1
Col1a2	collagen type 1, alpha 2
Col2a1	collagen type 2, alpha 1
CKIα	casein kinase Iα
Dkk1	dickkopf-1
EDTA	ethylenediaminetetraacetic acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSK-3β	glycogen synthase kinase 3β
H&E	hematoxylin and eosin
HBM	high bone-mass phenotype
i.p.	intraperitoneal

LD50	median lethal dose (lethal dose, 50%)
LEF	lymphoid enhancer-factor
LiCl	lithium chloride
Lrp5	low-density lipoprotein receptor-related protein 5
mA	milliamps
mmol/L	millimoles per liter
MSC	mesenchymal stem cell
NAC	N-acetylcystein
OCN	osteocalcin
OPG	osteoprotegerin
OPPG	osteoporosis-pseudoglioma syndrome
PBS	phosphate buffered saline
PGK-1	phosphoglycerate kinase-1
PPARγ	peroxisome proliferator-activated receptor gamma
pqCT	peripheral quantitative computed tomography
RANKL	receptor activator of nuclear factor kappa-B ligand
ROS	reactive oxygen species
Runx2	runt-related transcription factor 2
s.c.	subcutaneously
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sfrp4	secreted frizzled-related protein 4
SOST	sclerostin
Sox9	sex determining region Y-box 9
TBS	Tris-buffered saline
TCF	T cell factor
Ub	Ubiquitin
V	Volts
Vs.	versus
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactopyranos

CHAPTER I

INTRODUCTION

In 2006, approximately 3.8 million Americans entered the emergency department with a long bone fracture, and half of these cases required hospitalization (AAOS, 2008). Orthopaedic trauma of the extremities is a significant socioeconomic burden in the U.S., with more than $23 billion spent annually on fracture treatment costs, and an average hospital bill of $27,740 per patient (AAOS, 2008). In 2004 alone, bone fracture was responsible for over 1 million inpatient hospital stays (Agency for Healthcare Research and Quality, 2004). Underage drinking has become a serious issue, as alcohol-related traffic crashes and alcohol-fueled violence rank highest in associated economic burden as well as mortality (Hingson et al. 2002, Miller et al. 2006). Orthopaedic injuries account for a large percentage of all trauma-related injuries and 25-40% of all orthopaedic trauma patients present with positive blood alcohol levels (Levy et al. 1996). Many of these patients are not chronic alcoholics, but are instead under acute alcohol intoxication as a result of a binge-drinking episode, which has become the most common form of alcohol consumption among young adults (Centers for Disease Control and Prevention, 2012). These statistics denote the negative impact of alcohol consumption on the risk of sustaining a fracture, which may then lead to complications such as delayed union and

nonunion, conditions affecting 5-10% of fracture cases (Einhorn 1995). Although many clinical studies have associated alcohol and impaired fracture healing, there have been few studies that investigate the molecular effects of alcohol on deficient bone fracture repair. Alcohol abuse is a known risk factor for osteopenia and is also associated with increased fracture risk, delayed fracture healing, and increased incidence of impaired fracture healing and nonunion, conditions which require costly treatment interventions (Foulk and Szabo, 1995; Perlman and Thordarson, 1999; Mathog et al., 2000; Williams et al., 2008; Duckworth et al., 2011). Identifying molecular targets of alcohol in bone tissue would reveal potential therapies to improve fracture healing in at-risk individuals.

Fracture repair is a complex process beginning with the recruitment of stem cells to the site of injury and their differentiation into specialized cells that form a bridging callus to stabilize and initiate repair of the broken bone. Local and distant mesenchymal stem cells (MSC) differentiate into osteoblasts and chondrocytes to form new bone and cartilage at the site of injury. During canonical Wnt stimulation, tightly regulated levels of β-catenin play a pivotal role in the lineage commitment of MSC, since β-catenin promotes the expression of genes required for osteoblast and chondrocyte differentiation in bone tissue. Recent *in vivo* work has highlighted the significance of Wnt/β-catenin signaling during fracture repair, showing that precise regulation of β-catenin levels are required throughout healing in order to ensure union of the fracture and normal bone and cartilage development (Chen et al. 2007, Huang et al. 2012). Furthermore, our laboratory

has shown that binge alcohol exposure decreases the gene expression of a panel of canonical Wnt genes in uninjured rodent bone tissue, including β-catenin.

Collectively, these findings suggest that alcohol exposure may target the canonical Wnt signaling pathway during fracture repair, resulting in decreased bone and cartilage formation at the site of injury. These events will undoubtedly predispose alcohol-abusers to fracture-related complications such as delayed union or nonunion. The studies describedherein will determine the effect of alcohol on the canonical Wnt/β-catenin pathway during fracture repair to potentially reveal a connection between alcohol-induced β-catenin deregulation and impaired fracture healing. These findings will contribute to our understanding of the increased rate of complications observed in the alcohol-abusing population by identifying specific pathways targeted by alcohol exposure in healing bone tissue. Clinically, these data may also reveal therapeutic targets to aid in better care of musculoskeletal injuries, which are a significant healthcare burden in the United States.

Hypothesis: Binge alcohol exposure deregulates canonical Wnt signaling in the fracture callus, leading to impaired fracture healing.

Aim 1: To determine the effect of binge alcohol exposure on fracture callus strength, integrity, and cellular composition *in vivo*.

Aim 2: To investigate the effects of binge alcohol exposure on canonical Wnt signaling in the fracture callus by:

- Assessing levels of key pathway proteins
- Measuring downstream target gene activation

Aim 3: To determine if exogenous enhancement of the canonical Wnt pathway during healing can improve fracture repair in binge alcohol-treated mice.

CHAPTER II

REVIEW OF THE RELEVANT LITERATURE

ALCOHOL ABUSE AND ORTHOPAEDIC TRAUMA

In the United States alone, approximately 17.6 million people are considered alcoholics (National Institutes of Health). Within this population, the rate of fracture is up to four times higher than in non-abusers (Kristensson et al. 1980). Of the estimated 15 million fractures that are treated each year in the U.S. (United States Bone and Joint Decade, 2008), 5-10% result in delayed union or nonunion (Einhorn 1995, Marsh 1998), conditions requiring multiple costly and prolonged treatment interventions with varying success rates. In addition, between 25-40% of patients admitted with orthopaedic trauma screen positive for blood alcohol (Levy et al. 1996, Blake et al., 1997). Numerous clinical studies have associated alcohol abuse with a significantly increased risk of developing nonunion and delayed union (Foulk and Szabo, 1995; Perlman and Thordarson, 1999; Mathog et al., 2000; Williams et al., 2008; Duckworth et al., 2011).

Prevalence of Alcohol Abuse and Fracture

Binge drinking has become a prevalent form of alcohol consumption in the United States among all age groups. The National Institute on Alcohol Abuse and Alcoholism

defines binge drinking as a pattern of alcohol consumption that raises one's blood alcohol level to 0.08 grams percent or above during a single drinking episode. On average, this occurs when males consume 5 or more drinks and females consume 4 or more drinks in approximately 2 hours (Centers for Disease Control). In the United States alone, more than 38 million individuals, or 1 out of 6 people, binge drink approximately four times per month (Centers for Disease Control). Furthermore, adults consuming alcohol in a binge pattern consume on average 8 alcoholic drinks (Naimi et al. 2010), with the population aged 18-34 consuming the most drinks per episode (Centers for Disease Control). This intensity of binge drinking will consequently raise blood alcohol levels to well above the defined minimum of 0.08 grams percent. However, most binge drinkers are not physically dependent on alcohol (Centers for Disease Control) nor necessarily consume alcohol daily, and these characteristics define this population as unique from chronic alcoholics. This warrants separate studies to investigate the health impacts specifically related to this type of alcohol consumption.

Many clinical studies highlight the prevalence of acute alcohol intoxication and orthopaedic injury. One of the major causes of orthopaedic trauma are high-energy impact injuries, such as motor vehicle accidents. Over 500,000 alcohol-related motor vehicle accidents occur each year that result in death or injury (NHTSA 2008). Additionally, the vast majority of alcohol-impaired driving episodes involve binge drinkers (Flowers et al. 2008, Naimi et al. 2003). Intoxicated patients often experience more severe injuries and require longer hospital stays than non-intoxicated patients (Levy

et al. 1996), which contributes to the social and economic burden of alcohol abuse in the U.S.

Clinical Evidence for Alcohol Abuse and Impaired Fracture Healing

Another significant source of orthopaedic injury is from low-energy impacts such as falls, particularly within the osteoporotic population. Alcoholics frequently present with low bone mass or alcohol-induced osteoporosis (Bikle et al. 1985, Spencer et al. 1986, Peris et al. 1995), which further predisposes this population to suffering non-traumatic fractures. One of the earliest observations of fracture frequency in alcohol abusers noted that alcoholics present with a fracture pattern and history comparable to a population 20 years older (Snell 1971). Several studies since then have demonstrated that alcohol abuse is associated with a significantly increased risk and incidence of hip, wrist, and spinal fractures in both women (Clark et al. 2003, Tuppurainen et al. 1995, Hernandez-Avila et al. 1991, Paganini-Hill et al. 1981) and men (Felson et al. 1988, Santori et al. 2008), as well as decreased 1-year survival rates following hip fracture (Yuan et al. 2001).

Rodent Models of Alcohol Exposure and Impaired Fracture Healing

Despite numerous clinical evaluations of fracture healing complications and fracture risk in an alcoholic population, studying the effects of alcohol on specific bone healing parameters in humans, such as the biomechanical properties and tissue composition of the callus, presents a challenge. However, rat models have provided insight into how both chronic and acute alcohol exposure detrimentally affects fracture

repair. Rats receiving chronic alcohol treatment in the form of isocaloric liquid diets prior to fracture injury show delayed healing or nonunion, biomechanically weaker fracture callus tissue, and altered callus tissue composition with associated decreased bone mineral content (Nyquist et al. 1999, Elmali et al. 2002, Chakkalakal et al. 2005). In addition, many rat models that utilize distraction osteogenesis or osteotomy, a variation of fracture repair in which a small gap is created in the bone to observe rapid bone formation, report similar findings. These investigations showed that chronic alcohol exposure inhibits osteoinduction, impairs osteoblast-mediated mineralization of new bone, and decreases biomechanical strength compared to pair-fed control rats during bone repair (Trevisiol et al. 2007, Brown et al. 2002, Chakkalakal et al. 2002, Janicke-Lorenz and Lorenz 1984).

The vast majority of investigations have focused on the effects of chronic alcohol exposure on fracture repair. Studies from our laboratory have focused on acute binge alcohol exposure rodent models, which have demonstrated decreased biomechanical strength and bone mineral density in uninjured rats (Callaci et al. 2004). The binge alcohol paradigm exposes the rats to either 3 or 4 consecutive days of an intraperitoneal injection of an ethanol solution to mimic a binge-like drinking pattern. Utilizing this model, we have shown that binge alcohol exposure for 2 weeks results in a significant 53% reduction in biomechanical strength compared to saline-injected controls, and causes a replacement of normal, mature cartilaginous callus matrix with immature fibrous tissue at weeks 1, 2, and 3 post-fracture (Volkmer et al. 2011). These data were the first

to report that short-term binge alcohol treatment blunts normal fracture healing in rodents, yet a mechanism for alcohol's effects on healing remains unknown.

BONE HOMEOSTASIS AND THE EFFECTS OF ALCOHOL ON UNINJURED BONE

Clinical observations dating back to 1965 have reported on the negative effects of alcohol abuse on the skeleton in both men and women (Saville 1965). Alcoholics frequently present with osteopenia, or decreased bone mass (Bikle et al. 1985, Spencer et al. 1986, Garcia-Sanchez and Mundi 1999), and this bone loss can be of the same magnitude as that seen in postmenopausal females (Dalén and Lamke, 1976). Alcoholics also have an increased incidence and risk of suffering fractures compared to non-abusing individuals (Foulk and Szabo, 1995; Perlman and Thordarson, 1999; Mathog et al., 2000; Williams et al., 2008; Duckworth et al., 2011).

The Basics of Bone

As a result of these clinical observations, research has focused on the effects of alcohol on the cell types that participate in bone homeostasis; osteoblasts and osteoclasts. Bone is a metabolically active, specialized connective tissue that is constantly undergoing remodeling, which is a process that replaces old bone tissue with new bone tissue to adapt to mechanical load and strain.

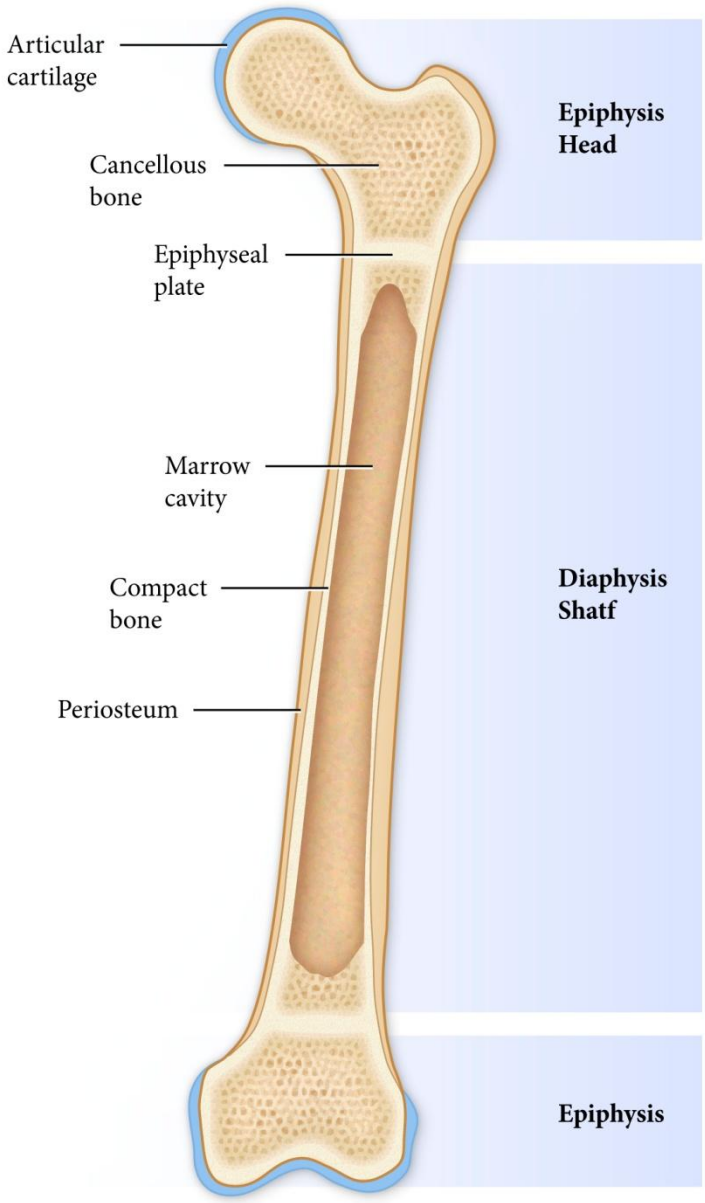

Figure 1. The anatomy of a long bone. The basic components of a long bone are outlined above. Osteoblasts and osteoclasts line the surfaces of the compact and cancellous bone tissue to create and resorb bone, Multipotent progenitor cells in the periosteum can differentiate into bone and cartilage cells, which is especially important following injury to the bone. (Image courtesy of Patrick Carrico)

There are two basic types of bone: an external layer of dense cortical (compact) bone that serves mainly for support and protection, and the inner cancellous (trabecular, spongy) bone, which also lends support and comprises the red marrow compartment in the ends of long bones. A dense connective tissue layer called the periosteum surrounds the external surface of the cortical bone and contains blood vessels that provide nutrients to the bone, fibroblasts, pericytes and multipotent progenitor cells with the capacity to differentiate into bone, cartilage, fat, and muscle (De Bari et al. 2006). The marrow cavity is lined by the endosteum, which also contains blood vessels, osteoclasts, and osteoblasts, and hematopoietic progenitor cells. The parts of a typical long bone and the location of cell types are outlined in **Figure 1.**

The two main cell types directly responsible for bone mass maintenance are the bone-resorbing osteoclasts, and bone-building osteoblasts. These cell types are organized into local bone remodeling units (BRU) or basic multicellular units (BMU), which maintain the continual sequence of osteoclast activation and resorption, followed by osteoblast infiltration and bone replacement (Frost et al. 1990). Osteoblasts secrete the unmineralized organic matrix known as osteoid, which consists of predominantly type I collagen in addition to proteoglycans, glycoproteins, and other noncollagenous proteins such as osteocalcin (Anderson 1989). Osteoblasts also secrete enzymes such as alkaline phosphatase, which are essential for proper matrix mineralization and serves as a marker of osteoblast activation and maturation (Anderson et al. 2004). Osteoblasts control the development of osteoclasts by secreting receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG), factors which promote maturation or

inhibition of osteoclast differentiation respectively (Nakagawa et al. 1998), and therefore can directly restrict excessive resorption. As described in the following sections, the gene expression of many essential osteoblast and osteoclast proteins are regulated by canonical Wnt signaling.

The resorptive phase of the bone remodeling cycle occurs in a shorter amount of time and precedes the much slower bone formation phase, creating a tightly coupled and highly regulated balance between the two phases in order to maintain bone mass. Prior to adulthood, the bone remodeling cycle becomes uncoupled to favor bone formation, resulting in the accrual of 90% of peak bone mass between the ages of 20 to 25. Bone mineral density plateaus in adulthood, then eventually begins to decrease with age and gonadal insufficiency, when uncoupling of the bone remodeling cycle favors increased resorption.

Alcohol Exposure and Bone Loss

Alcohol abuse directly impacts the maintenance of the bone remodeling cycle, and more specifically, bone formation. Serum bone formation markers such as osteocalcin have been shown to be decreased following both acute and chronic alcohol exposure in humans (Garcia-Sanchez et al. 1995, Nyquist et al. 1996, Gonzalez-Calvin et al.1999, Jaouhari et al. 1993) while some studies have reported increased serum bone resorption markers (Nyquist et al. 1996, Diez-Ruiz et al. 2010). Furthermore, osteoblast number and synthesis of osteoid is significantly reduced in alcoholics (Crilly et al. 1988, Chappard et al. 1991), suggesting that alcohol abuse suppresses osteoblast activity and bone formation, resulting in net bone loss. Due to the deleterious effects of heavy

drinking on bone formation, it has been postulated that young binge drinkers may put themselves at risk for future osteopenia and increased fractures, since heavy episodic drinking may compromise the ability to reach peak potential bone mass.

Rodent models have provided valuable insight on mechanisms of alcohol-induced bone loss. Many studies have shown that rats receiving chronic alcohol in the form of a liquid diet (Lieber and DeCarli, 1982) demonstrates significantly decreased cortical and trabecular bone mineral density and decreased biomechanical strength (Sampson et al. 1996, Nishiguchi et al. 2000, Nyquist et al. 2002, Wezeman et al. 2003). Chronic alcohol exposure in rats also causes decreased osteoblast proliferation, activity, and gene expression *in vivo* (Dyer et al. 1998, Sampson 1998, Wezeman et al. 1999), which supports that alcohol exposure leads to bone loss through suppression of osteoblast function.

The acute binge alcohol exposure model developed in our laboratory demonstrates significantly reduced trabecular bone mineral density (BMD) and vertebral compressive strength in adult rats (Callaci et al. 2004, 2006, 2009). In contrast to models of chronic alcohol exposure, only trabecular bone appears to be decreased following acute binge alcohol exposure, while no change is seen in cortical bone. Adolescent-stage rats also demonstrate significantly decreased cancellous BMD and vertebral compressive strength following binge alcohol exposure. However, a 30-day period of alcohol abstinence does not completely restore vertebral BMD or strength in these rats, suggesting that alcohol abuse during critical growth periods may result in persistent, irreversible changes to the skeleton (Lauing et al. 2008). Additionally, acute binge

alcohol exposure transiently decreases serum osteocalcin levels in rats (Lauing et al. 2008), which again implicates that binge alcohol also targets osteoblast function.

In addition to *in vivo* reports of alcohol-induced suppression of osteoblast function and number, *in vitro* studies have provided insight into the direct effects of alcohol on osteoblast differentiation. In human bone marrow-derived mesenchymal stem cells (MSC), the common progenitor of adipocytes, osteoblasts and chondrocyte, *in vitro* exposure to alcohol following osteogenic induction decreases the expression of type I collagen, which constitutes 90% of the bone matrix. Additionally, alcohol exposure suppressed markers of osteogenesis in MSC while promoting adipocyte formation (Wang et al. 2003, Gong and Wezeman 2004, Wezeman and Gong 2004, Cui et al. 2006). Similarly to *in vivo* findings, co-culture of alcohol and human osteoblastic cells results in reduced proliferation, decreased osteocalcin, and decreased alkaline phosphatase produced by active osteoblasts (Friday and Howard 1991, Chavassieux et al. 1993). However, alcohol does not promote osteoblast apoptosis, indicating that the effects of alcohol on osteoblasts are antiproliferative rather than toxic (Klein 1996). Collectively, the *in vitro* studies support that alcohol exposure shifts the differentiation potential of MSC toward adipogenesis at the expense of osteogenesis, and also directly impacts osteoblast proliferation and function.

Taken together, the data show that alcohol exposure reduces markers of bone formation and decreases biomechanical strength and bone mineral density *in vivo.* I*n vitro* studies have shown this may be due to direct effects of alcohol on osteoblast proliferation, differentiation, and function. A better understanding of specific pathways

that control osteoblast function and differentiation, such as the canonical Wnt pathway, could provide insight into the mechanism of action of alcohol in bone loss and injury.

THE CANONICAL WNT PATHWAY

Within the last decade, canonical Wnt signaling has emerged as a powerful regulator of normal bone metabolism. The link between canonical Wnt signaling and bone mass was first discovered when loss- or gain-of-function mutations in the Wnt co-receptor low-density lipoprotein receptor 5 (Lrp5) were the cause of two rare skeletal disorders, osteoporosis-pseudoglioma (OPPG) or high bone-mass phenotype (HBM) (Gong et al. 2001, Boyden et al. 2002). Since this discovery, the role of canonical Wnt signaling in normal bone homeostasis has been extensively studied. Canonical Wnt signaling is not only directly associated with bone formation and osteogenesis, but has also recently shown to be tightly regulated during fracture repair (Chen et al. 2007, Huang et al. 2012). An overview of the canonical Wnt pathway is illustrated in **Figure 2.**

Wnts are a family of highly conserved, hydrophobic glycoproteins that are secreted and stimulate the canonical and/or noncanonical pathways (Willert et al. 2003). Nineteen Wnts are currently characterized, and are classified as "canonical" based on their ability to stimulate β-catenin nuclear localization. Two canonical Wnts in particular, Wnt 3a and Wnt 10b, are especially important in inhibiting adipogenesis,

promoting osteogenesis, and inducing osteoprogenitor proliferation from mesenchymal precursors (de Boer et al. 2004, Bennett et al. 2005).

In the absence of Wnt stimulation, β-catenin proteins that are not involved with the cadherin complexes are rapidly phosphorylated and targeted for ubiquitin-mediated proteosomal degradation (**Figure 2A,** Aberle et al. 1997). A large cytosolic complex involving multiple proteins serves as the negative regulation complex of the pathway. The scaffolding protein Axin interacts directly with adenomatous polyposis coli (APC) and brings glycogen synthase kinase (GSK) 3β and casein kinase (CK) I into proximity to the core destruction complex (Ikeda et al. 1998, Liu et al. 2002). When this complex is assembled, activated CK1 phosphorylates β-catenin at serine 45, followed by sequential phosphorylation at threonine 41, serine 37, and serine 33 by activated GSK-3β (Orford et al. 1997, Liu et al. 2002). These phosphorylation events result in β-catenin binding to the E3 ubiquitin ligase β-Trcp, and proteasomal degradation (Hart et al. 1999, Winston et al. 1999). To avoid excessive stimulation or to inhibit the pathway, there are also several endogenous inhibitors that suppress Wnt signaling by functioning extracellularly. Dickkopf-1 (Dkk1) and sclerostin (SOST) prevent β-catenin activation by directly binding the Lrp5/6 coreceptor and preventing its association with Wnt-bound Frizzled (Li et al. 2005, Semënov et al. 2008), while secreted frizzled related proteins (SFRP) bind soluble Wnts to compete with and intercept binding to Frizzled/Lrp5/6 (Dennis et al. 1999).

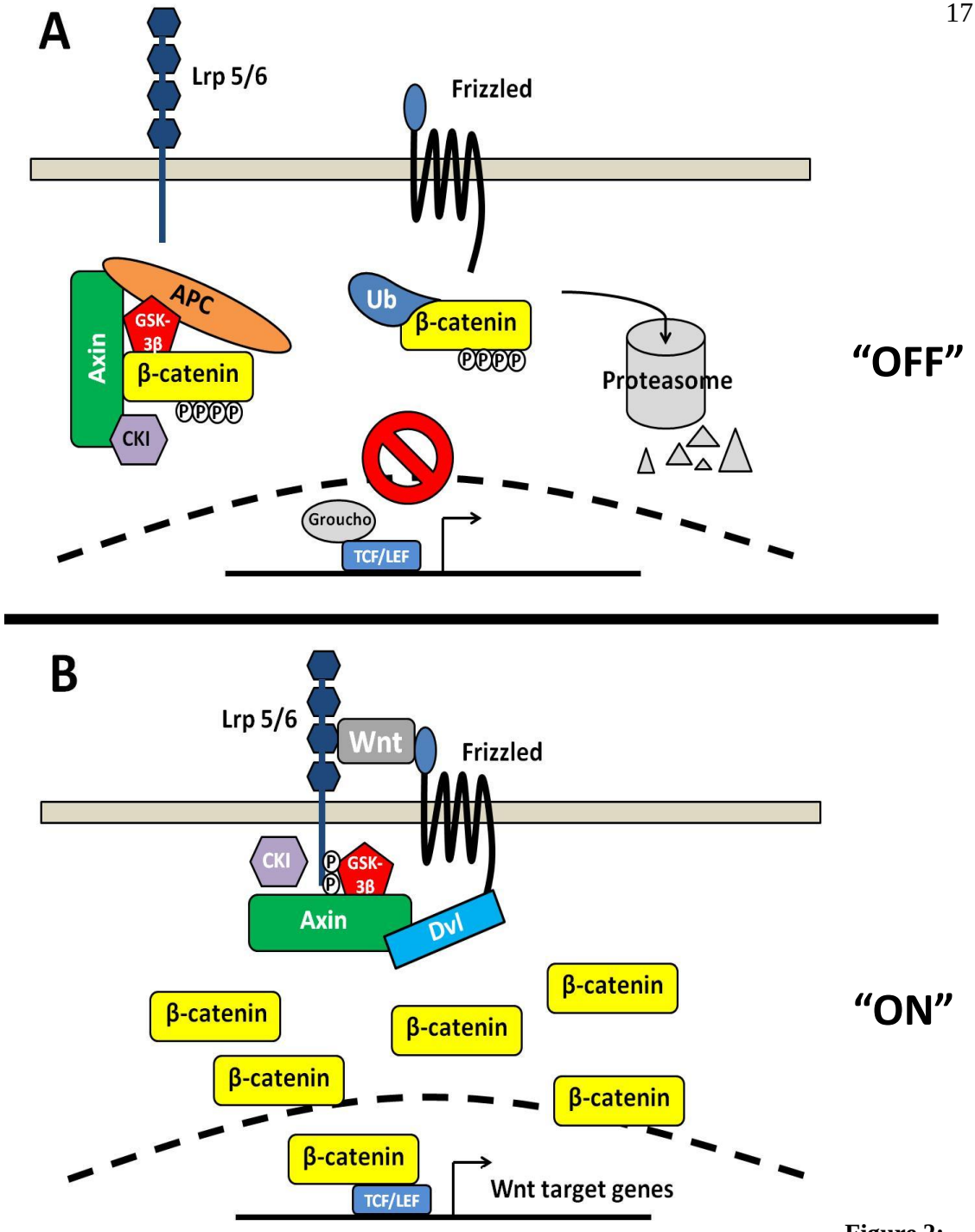

Figure 2:

Overview of the canonical Wnt pathway. A). When Wnt proteins are absent, β-catenin is rapidly degraded by a destruction complex formed by Axin, APC, and GSK-3β. β-catenin is phosphorylated by CK1, then by GSK-3β, which promotes its ubiquitination (Ub) and proteasomal degradation. B). Wnt proteins, when bound to Frizzled, promote β-catenin stabilization. Wnt stimulation induces dissociation of the destruction complex through Dishevelled (Dvl), which prevents β-catenin hyperphosphorylation by GSK-3β. β-catenin

translocates to the nucleus and displaces transcriptional repressors (Groucho) to induce Wnt target gene expression through TCF/LEF binding.

In the pathway's activated state, Wnt proteins bind to seven-transmembrane domain Frizzled receptors, and associate with the coreceptor Lrp5/6 on the cell membrane (Bhanot et al. 1996, Wehrli et al. 2000, **Figure 2B**). The Wnt signal is transmitted intracellularly via the protein Dishevelled (Dvl) (Noordermeer et al. 1994), which recruits axin to the intracellular tail of Lrp5 (Tamai et al. 2004), disrupts the necessary binding of GSK-3β to axin, and ultimately prevents β-catenin phosphorylation (Ikeda et al. 1998). This effectively dismantles the negative regulatory complex of the Wnt pathway and results in the accumulation and increased half-life of β-catenin in the cytoplasm (Staal et al. 2002). Following cytoplasmic stabilization of β-catenin by Wnt stimuli, the Armadillo (arm) repeats within the central structure of β-catenin facilitate nuclear transport by binding to nucleoporin complexes (Sharma et al. 2012). In the nucleus, the C-terminus of β-catenin binds and forms heterodimers with the T cell factor/lymphoid enhancer-binding factor (TCF/LEF) family of transcription factors through displacement of transcriptional repressors such as Groucho (Behrens et al. 1996, Molenaar et al. 1996, Daniels and Weis 2005). β-catenin/TCF-driven transcription plays a key role in bone homeostasis and repair, as discussed in the following sections.

BONE HOMEOSTASIS AND CANONICAL WNT SIGNALING

Mesenchymal Stem Cell Differentiation and Canonical Wnt Signaling

The β-catenin/TCF/LEF heterodimers are known to initiate transcription of a wide variety of target genes essential for cell differentiation. The Wnt pathway is autoregulated, therefore upon activation of the pathway, the transcription of negative regulators of the pathway and endogenous inhibitors such as Axin2 and Dickkopf-1 (Dkk1) are induced (Jho et al. 2002, Chamorrow et al. 2005). In bone tissue, Wnt stimulation and β-catenin/TCF-dependent transcription are involved in the expression of several genes required for osteoblast and chondrocyte differentiation such as Runx2 and Sox9 (Hill et al. 2005, Dong et al. 2006), and osteoblast function such as osteocalcin and alkaline phosphatase (Rawadi et al. 2003, Gaur et al. 2005). These genes are tightly regulated by canonical Wnt signaling and essential for mesenchymal stem cell (MSC) differentiation into bone and cartilage.

Osteoblasts and chondrocytes are both derived from MSC, which are a readily-available, multipotent, self-renewing cell population that makes them ideal targets for tissue engineering and gene therapies. MSC reside in the stromal compartment of the bone marrow but can be isolated from several other tissues, and are capable of differentiating into osteoblasts, chondrocytes, adipocytes, or myocytes, given certain culture conditions (Baksh et al. 2004). Decades ago, it was noted that a reciprocal relationship between osteogenesis and adipogenesis exists in bone marrow stromal cell cultures (Beresford et al. 1992), and it was later attributed to the canonical Wnt/β-catenin

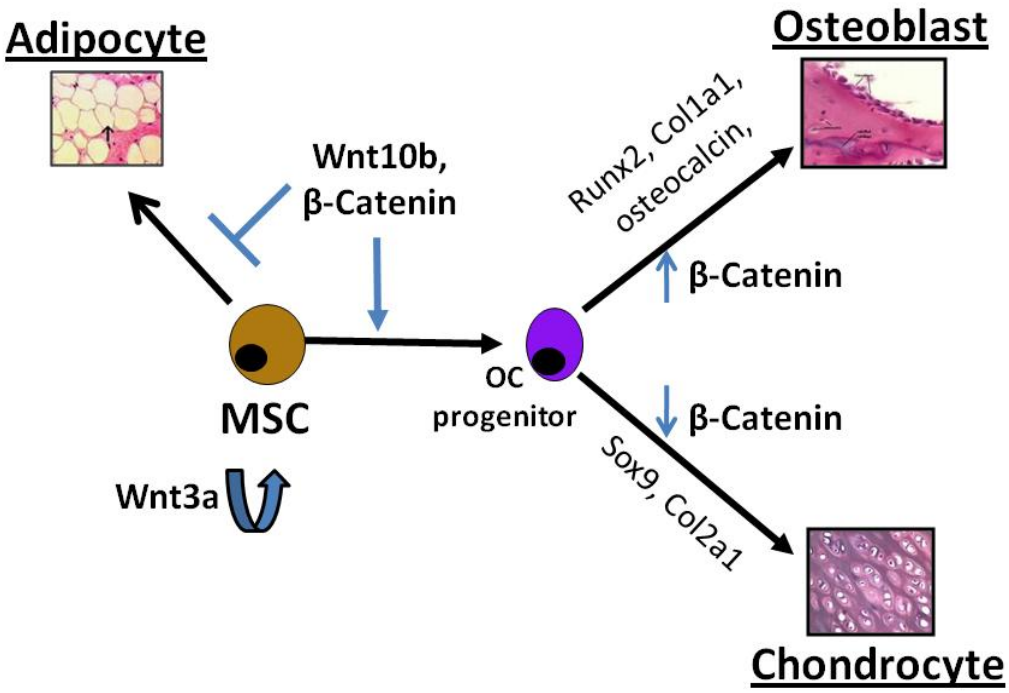

Figure 3. **Wnt/β-catenin control of mesenchymal stem cell differentiation.** Stabilization of β-catenin is essential to push mesenchymal stem cells (MSC) toward the osteo-chondroprogenitor (OC) lineage. Wnt stimulation, particularly by Wnt10b, and β-catenin stabilization inhibits the differentiation of adipocytes. Wnt3a maintains MSC and OC progenitor populations when present at high levels. β-catenin is also essential for the expression of several genes required for osteoblast and chondrocyte differentiation, and therefore plays a central role in fracture repair. Low levels of β-catenin allow chondrocyte development from OC progenitors, while high levels of β-catenin promote osteoblast differentiation and function.

pathway that regulates this inverse relationship and plays a central role in the lineage commitment of MSC into osteochondroprogenitors (**Figure 3**).

Canonical Wnt signaling is necessary for MSC proliferation and self-renewal though this effect is dose-dependent and highly regulated, as excessive signaling can

inhibit proliferation (De Boer et al. 2004). Wnt3a has been characterized as the predominant canonical Wnt protein involved in both human and rodent MSC proliferation and renewal (Boland et al. 2004, Derfoul et al. 2004), though some reports show that certain levels of Wnt3a can induce osteoblast formation and promote mature chondrocyte proliferation (Zhou et al. 2008, Yasuhara et al. 2011). Wnt10b expression in mesenchymal precursors potently suppresses markers of adipocyte formation, such as peroxisome proliferator-activated receptor gamma (PPARγ) while rapidly increasing markers of osteoblast formation (Bennett et al. 2005, Kang et al. 2007). Knockdown or inhibition of β-catenin in MSC prevents Wnt-mediated inhibition of adipogenesis, which demonstrates the pivotal role that Wnt/β-catenin signaling plays in MSC fate (Cawthorn et al. 2012, Li et al. 2008).

The canonical Wnt pathway also controls chondrogenic differentiation and hypertrophic maturation through the expression of Sox9 (Akiyama et al. 2004, Yano et al. 2005), though the timing of increased β-catenin expression in osteo-chondroprogenitors is crucial to determine whether the cell becomes an osteoblast or chondrocyte (Day et al. 2005). The presence of increased stabilized β-catenin has been shown to maintain chondrocytes in a proliferative phenotype, whereas ablation of β-catenin in chondrocytes causes accelerated maturation and hypertrophy (Yasuhara et al. 2011). Increased ectopic Wnt/β-catenin signaling at crucial points in growth plate development of bones causes phenotypic regression of chondrocytes and joint deformities (Yuasa et al. 2009), indicating that increased β-catenin stabilization is detrimental during early chondrocyte development. In summary, the data demonstrate that chondrocyte and osteoblast

differentiation from MSC requires precise levels of Wnt/β-catenin signaling for normal development and function, and the perturbation of these levels during development can result in abnormal bone and cartilage formation.

Canonical Wnt Signaling Defects and Bone Abnormalities

Clinically, defective Wnt signaling is observed in several bone diseases. As mentioned previously, rare bone mass disorders resulting from mutations in the Lrp5 co-receptor were the first reports to link canonical Wnt signaling and bone homeostasis. Patients with the loss-of-function mutation are severely osteopenic while patients with the gain-of-function mutation have significantly increased bone mass and cortical bone thickness (Gong et al. 2001, Boyden et al. 2002). Mutations in APC, which are the cause of diseases like familial adenomatous polyposis, are also associated with increased bone mass due to the inability of APC to bind and activate the β-catenin destruction complex, leading to constitutive activation of the Wnt pathway (Miclea et al. 2010). Defects in the osteocyte-secreted Wnt inhibitor sclerostin (SOST) result in two rare recessive bone disorders, sclerosteosis and van Buchem disease, which are characterized by excessive bone formation and bone overgrowth mainly in the skull (Balemans et al. 2001, 2002), implicating the significant role of the negative regulators of the pathway as well.

Genetic manipulations of the pathway in rodents provide further insight into the significance of canonical Wnt signaling in bone mass maintenance. Germ-line knockouts of several pathway components, such as APC, Dkk1, GSK-3β, result in embryonic or perinatal lethality (Moser et al. 1995, Hoeflich et al. 2000, Mukhopadhyay et al. 2001),

highlighting the essential role of Wnt signaling proteins in embryonic and skeletal development. As a result, heterozygous or conditional knockout approaches have served as valuable models with which to study specific roles of each protein in skeletal development. In humans the high bone mass (HBM) or osteoporotic phenotypes associated with the gain- and loss-of-function of Lrp5 respectively, can be recapitulated in mice overexpressing the receptor containing the human mutation and in *Lrp5-/-* mice. These models revealed that Lrp5 is expressed on cells of the osteoblast lineage and is crucial for osteoblast proliferation and function (Kato et al. 2002). Likewise, stabilization of β-catenin specifically in osteoblasts results in extremely high bone mass and reiterates the HBM phenotype, while osteoblast-specific deletion of β-catenin leads to osteopenia (Glass et al. 2006). Additionally, mice deficient in Wnt10b display increased biomechanical strength of long bones and maintain increased bone mass throughout 23 months of age (Bennett et al. 2005). Collectively, studies describing the skeletal effects of deficiencies in canonical Wnt pathway proteins demonstrate the importance of Wnt/β-catenin regulation in promoting bone homeostasis and normal bone and cartilage formation.

Interestingly, we have observed that binge alcohol treatment significantly decreases the expression of β-catenin and Lrp5 in rat lumbar vertebrae (Himes et al. 2008, Callaci et al. 2010), suggesting that alcohol exposure targets the canonical Wnt pathway in uninjured bone. These findings were confirmed by a separate group that also observed an alcohol-associated suppression of canonical Wnt signaling in bone, including a significant decrease in β-catenin and Lrp5, while alcohol exposure resulted in a

significant increase in the endogenous inhibitor Dkk1 (Chen et al. 2010). However, the effects of alcohol on the Wnt signaling pathway during fracture repair have not been investigated, and would help clarify our understanding of the mechanisms behind alcohol-related complications in orthopaedic injury.

REGULATION OF FRACTURE REPAIR BY CANONICAL WNT SIGNALING

The Phases of Fracture Healing

Fracture repair is a highly regulated and coordinated process that requires the differentiation of bone and cartilage at the injury site from local and distant MSC and osteoprogenitors. Fracture healing can be divided into 4 basic stages: (1) inflammatory response; (2) primary cartilaginous callus formation; (3) secondary bony callus formation; and (4) remodeling (**Figure 4;** reviewed in Chen and Alman, 2009 and Einhorn 1998). The majority of fractures generally heal through these sequential steps, which is also known as indirect healing. Prior to callus formation, the inflammatory response triggers the development of a hematoma, or blood clot, to halt bleeding that occurred from vascular injury. The hematoma formation leads to the recruitment and proliferation of undifferentiated MSC at the site of fracture, and the subsequent formation of granulation tissue. This tissue supports the subsequent differentiation of MSC into chondrocytes and osteoblasts, which synthesize the cartilage and bone that comprise the fracture callus tissue (Bolander 1992).

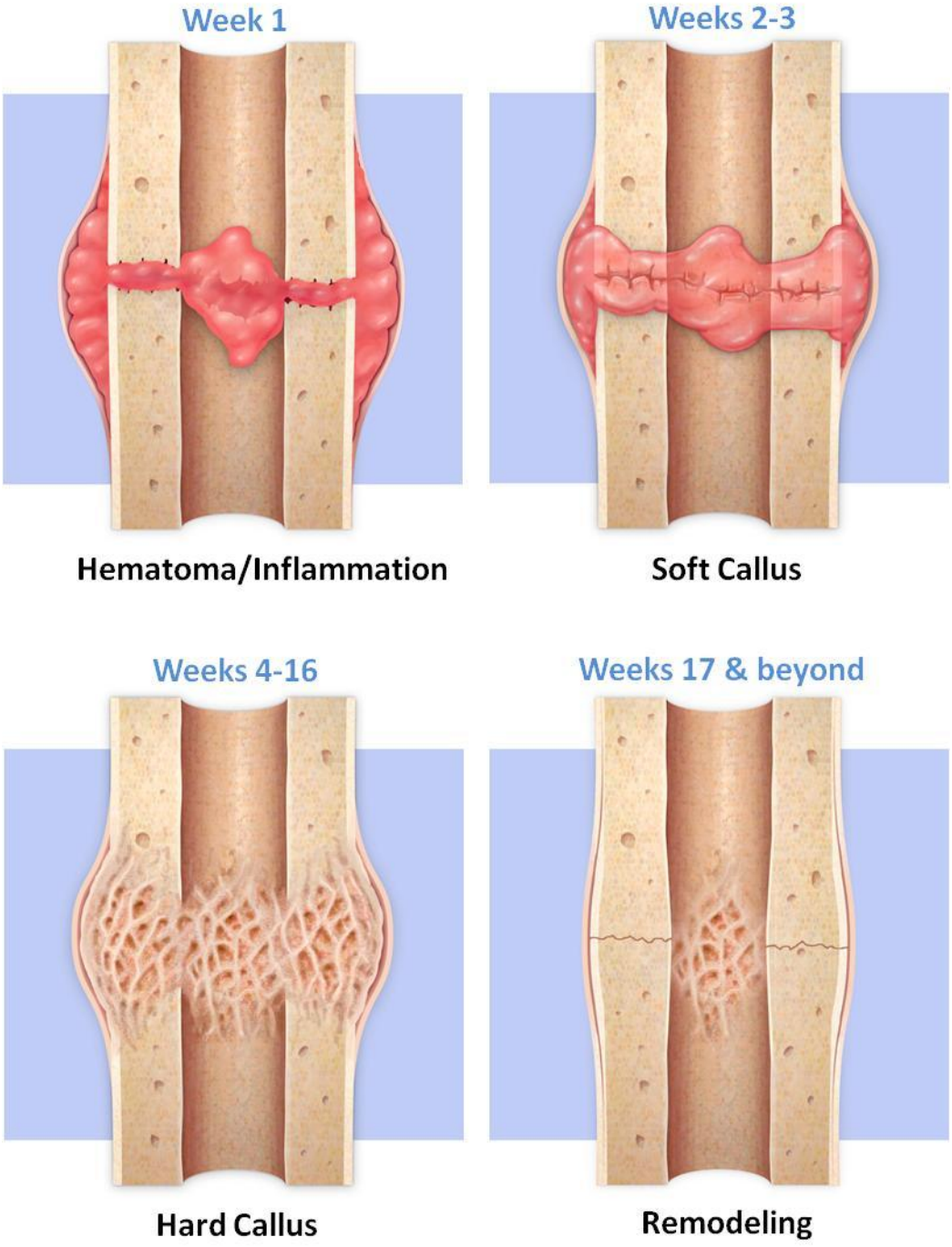

Figure 4: **Overview of the fracture repair process.** Fracture healing occurs in four main overlapping phases: (1) the inflammatory phase (2) cartilage formation from local and distant mesenchymal stem cells (3) replacement of cartilage with bone via endochondral ossification (4) remodeling phase. The time course for each stage of healing shown here is representative of human fractures; the course of healing in mice is at least twice as fast as that in humans. (Image courtesy of Patrick Carrico)

Bone formation in stabilized fractures occurs via two ways: (1) direct bone formation by osteoblasts, or intramembranous ossification, and (2) indirect bone formation, or endochondral ossification, which begins with a cartilage precursor. The periosteal callus (anchoring callus) and the intramedullary callus tissue are formed by direct bone formation, since both the periosteum and endosteum layers of bone contain osteoprogenitors that begin differentiation into osteoblasts immediately following the injury. These osteoblasts begin laying down osteoid at the distal ends of the injury site and within the marrow space to initiate stabilization of the injury. The external callus tissue is the majority of the tissue surrounding the fracture site, and is first formed by the differentiation of local and distant MSC into chondrocytes to form the soft callus. Once the bone has been mechanically stabilized, the process of endochondral ossification is initiated, and the cartilage is replaced by woven bone. This occurs when the cartilage matrix is infiltrated by blood vessels, which allow osteoblasts to begin mineralizing the cartilage matrix. In order for bone formation to occur at the fracture site, endochondral ossification is a critical and necessary event during bone healing.

Importance of β-catenin Regulation During Fracture Healing

During fracture repair, β-catenin levels must be tightly regulated throughout healing to promote osteo-chondroprogenitor differentiation from MSC and, to regulate the function of mature osteoblasts, and to direct the process of endochondral ossification. Chen et al. (2007) were the first group to show that there is a robust increase in β-catenin expression following fracture injury, which is associated with β-catenin/TCF-mediated

transcriptional activation. They observed that transient stabilization of β-catenin or deletion in the fracture callus tissue resulted in malunion or delayed union, demonstrating that precise regulation of β-catenin in fracture callus tissue is required for normal healing. Furthermore, these observations were associated with a significant decrease in the expression of the required osteoblast and chondrocyte transcription factors that are induced upon Wnt stimulation, Sox9 and Runx2. Interestingly, this study also noted that treatment with the GSK-3β inhibitor, lithium chloride (LiCl), at 4 days post-fracture could accelerate healing and bone formation at the fracture site, while treatment with LiCl earlier in fracture repair caused decreased bone formation in the fracture callus (Chen et al. 2007). This investigation was pivotal in establishing the requirement for strict Wnt/β-catenin pathway regulation during fracture healing to promote bone and cartilage formation at the injury site.

The regulation of Wnt/β-catenin has been shown to be essential for the process of endochondral ossification and chondrocyte maturation in growth plates and in the cartilage of fracture callus tissue. Deletion of β-catenin/TCF signaling specifically in chondrocytes leads to delayed fracture healing and decreased biomechanical strength of the callus tissue (Huang et al. 2012). The impaired healing observed in this model is due to the disruption of endochondral ossification in the fracture callus, and is coupled with altered expression of Runx2 and Sox9. Other investigations have implicated Wnt/β-catenin signaling as the primary regulator of chondrocyte hypertrophy in zones of endochondral ossification and of cartilage phenotype (Kitagaki et al. 2003, Tamamura et al. 2005, Zhong et al. 2006). These studies demonstrate that Wnt/β-catenin signaling is

highly active in chondrocytes during endochondral ossification, and perturbation of Wnt/β-catenin during the critical maturation stages of chondrocytes inhibits this process.

Canonical Wnt Inhibitors and Fracture Repair

Selective inhibition of endogenous canonical Wnt inhibitors can indirectly stimulate the pathway during fracture healing, and may be valuable therapeutic targets for fracture complications. The germ-line deletion of the Wnt inhibitor Dkk1 in mice results in embryonic lethality (Mukhopadhyay et al. 2001), however treatment of fractured mice with antibodies against Dkk1 greatly improves the repair process in wild type mice, as well as in osteopenic mice lacking Lrp5 co-receptor (Chen et al. 2007, Komatsu et al. 2010). Mice deficient in sFRP1show accelerated healing due to increased direct bone formation, suggesting that deletion of this canonical Wnt inhibitor causes a shift in stem cell differentiation toward the osteoblast lineage (Gaur et al. 2009). In addition, mice treated with monoclonal antibodies against (SOST) also show increased bone formation at the fracture site and improved healing compared to wild-type mice (Ominsky et al. 2011).

Together, these data implicate the canonical Wnt signaling pathway as highly regulated pathway that directs the fracture repair process by promoting bone and cartilage differentiation at the site of injury and initiating endochondral ossification. Individuals at risk for fracture-related complications, such the alcohol-abusing population, may benefit from canonical Wnt pathway enhancement to improve fracture healing.

WNT SIGNALING AND ALCOHOL EXPOSURE IN BONE

There are only a few studies that report that alcohol exposure decreases osteogenesis through inhibition of the Wnt pathway and β-catenin nuclear localization (Yeh et al. 2008, Chen et al. 2010). Chen et al. (2010) showed in osteoblastic cells that the alcohol-associated decrease of overall levels of β-catenin and its subsequent nuclear localization following was due to increased GSK-3β activation. These effects were reversed by treatment with a potent antioxidant, suggesting that alcohol exposure induces oxidative stress in osteoblasts. In addition, this study reported an overall suppression of canonical Wnt pathway genes *in vivo* following chronic alcohol exposure, including Axin, β-catenin, Lrp5, and GSK-3β. *In vitro* alcohol exposure to human bone marrow cells demonstrated an alcohol-related decrease in β-catenin nuclear localization and a concomitant increase in makers of adipocyte formation (Yeh et al. 2008). These data suggest that the shift in differentiation potential of human mesenchymal stem cells toward the adipogenic lineage following alcohol exposure is due to perturbations in the canonical Wnt signaling pathway during MSC differentiation. Further studies are warranted to investigate whether alcohol exposure prior to fracture injury deregulates canonical Wnt signaling at the fracture site, and whether this contributes to the increased frequency of impaired healing observed in individuals abusing alcohol.

CHAPTER III

ACUTE BINGE ALCOHOL EXPOSURE IMPAIRS FRACTURE HEALING AND DEREGULATES β-CATENIN SIGNALING IN THE FRACTURE CALLUS

ABSTRACT

Background: Alcohol abuse is a risk factor for bone damage and fracture-related complications. Through precise β-catenin signaling, canonical Wnt signaling plays a key role in fracture repair by promoting the differentiation of new bone and cartilage cells. In this study, we examined the effects of alcohol on the Wnt pathway in injured bone using a murine model of alcohol-induced impaired fracture healing.

Methods: Male C57Bl/6 or TCF-transgenic mice were administered 3 consecutive daily intraperitoneal doses of alcohol or saline. For biomechanical studies, mice were given 3 consecutive doses, left undisturbed for 4 days, then given an additional 3 consecutive daily injections for a total of 6 intraperitoneal doses. One hour following the final injection, mice were subjected to a stabilized, mid-shaft tibial fracture. Injured and contralateral tibias were harvested at 6, 9, or 14 days post-fracture for analysis of fracture callus size, biomechanical strength, tissue composition, and activation of the Wnt/β-catenin pathway.

Results: Acute binge alcohol treatment was associated with a significant decrease in fracture callus volume, diameter, and biomechanical strength at day 14 post-fracture. Histology revealed an alcohol-related reduction in cartilage and bone formation at the

fracture site, and that alcohol inhibited normal cartilage maturation. Acute alcohol exposure caused a significant 2.3-fold increase in total β-catenin protein at day 6 and a significant decrease of 53% and 56% at days 9 and 14 respectively. *LacZ* staining in β-galactosidase-expressing TCF-transgenic mice revealed spatial and quantitative differences in Wnt-specific transcriptional activation at day 6 in the alcohol group. Days 9 and 14 post-fracture showed that acute alcohol exposure decreased Wnt transcriptional activation, which correlates with the modulation of total β-catenin protein levels observed at these time points.

Conclusions: Acute alcohol exposure resulted in significant impairment of fracture callus tissue formation, perturbation of the key Wnt pathway protein β-catenin, and disruption of normal Wnt-mediated transcription. These data suggest that the canonical Wnt pathway is a target of alcohol in bone, and may partially explain why impaired fracture healing is observed in alcohol-abusing individuals.

INTRODUCTION

Fracture nonunion, or cessation of bone healing without bridging, occurs in 5-10% of the estimated 13 million fractures that are treated annually in the U.S. (Einhorn, 1995; Marsh, 1998; AAOS, 2008). The rate of fracture among alcoholics is up to four times higher than in non-abusers (Kristensson et al., 1980), and alcoholics frequently present with osteopenia or osteoporosis (Bikle et al., 1985, Spencer et al. 1986). Up to 40% of patients presenting with orthopaedic trauma are acutely intoxicated, highlighting

the prevalence of binge alcohol consumption and incidence of fracture (Levy et al., 1996; Blake et al., 1997). Numerous clinical studies have associated alcohol abuse with a significantly increased risk of developing nonunion and delayed union (Foulk and Szabo, 1995; Perlman and Thordarson, 1999; Mathog et al., 2000; Williams et al., 2008; Duckworth et al., 2011), and these conditions require costly and prolonged treatment options. Several animal studies using models of alcohol exposure and bone injury also report delayed healing and lack of fracture callus formation (Jänicke-Lorenz and Lorenz, 1984; Chakkalakal et al., 2005; Trevisiol et al., 2007). However, a mechanism for-induced impaired fracture healing has not yet been characterized. Fracture repair requires the mobilization and differentiation of mesenchymal stem cells and osteoblast precursors from local and distant niches to the site of injury (Devine et al., 2002; Shen et al. 2002; Taguchi et al., 2005; Kumagai et al. 2008). The initiation of repair requires differentiation of mesenchymal stem cells into bone and cartilage-forming cells, a process that is tightly controlled by canonical Wnt/β-catenin signaling (Day et al., 2005; Hill et al. 2005; Baksh et al., 2007). Canonical Wnt signaling has emerged as a complex pathway that is tightly regulated during bone repair and appears to control the fracture repair process (Chen et al., 2007; Komatsu et al. 2010; Huang et al. 2011). In the absence of pathway stimulation, stabilized β-catenin levels in bone tissue are kept low by a destruction complex consisting of APC, Axin, and glycogen synthase kinase 3-beta (GSK-3β), which promotes GSK-3β-mediated phosphorylation of β-catenin on specific residues that lead to its proteasomal degradation. During activation of the pathway, Wnt proteins bind to Frizzled/Lrp5/6 receptors on the cell membrane, causing disintegration of

the destruction complex and accumulation of β-catenin in the cytosol. β-catenin then translocates to the nucleus and promotes target gene transcription by binding to T cell factor/lymphoid enhancer-binding factor (TCF/LEF) family of transcription factors (reviewed in Kikuchi, 2000).

Recent studies have emphasized the importance of β-catenin and canonical Wnt signaling in osteogenesis and chondrogenesis by highlighting that precise levels of β-catenin stabilization are required during different stages of mesenchymal stem cell differentiation toward the osteoblast and chondrocyte lineage (Day et al., 2005; Silkstone et al., 2008; Miclea et al., 2009). Furthermore, deletion of β-catenin in osteoblasts or chondrocytes in mouse models of fracture repair in the tibia have been shown to cause delayed union and decreased bone and cartilage formation within the fracture callus (Chen et al., 2007, Huang et al. 2011). To date, no studies have examined whether alcohol exposure causes disruption of this pathway or β-catenin stabilization during fracture repair, which would contribute to our understanding of fracture-related complications seen in alcohol-abusing individuals.

Our laboratory has previously published data in rodents showing acute alcohol exposure modulates the expression of a number of genes belonging to the canonical Wnt pathway in bone tissue, including β-catenin (Himes et al., 2008; Callaci et al. 2010). Therefore, using a mouse model of acute alcohol exposure and tibial fracture, we sought to determine whether a). acute alcohol exposure impairs normal fracture healing and b). if alcohol exposure causes disruption of Wnt/β-catenin signaling in the fracture callus during the repair process. For the current investigation, we hypothesized that acute

alcohol exposure prior to orthopaedic injury would lead to decreased bone healing and cause deregulation of β-catenin levels during fracture repair.

RESULTS

1. Effects of Acute Alcohol on Fracture Callus Size and Volume

The method of binge alcohol administration utilized in the following experiments (outlined in detail in Chapter VI) resulted in peak blood alcohol concentrations of approximately 300 mg/dl at 30 minutes post-intraperitoneal (i.p.) injection. One hour after i.p. injection, which corresponds to the time of fracture injury, the mice averaged a blood alcohol concentration of approximately 200 mg/dl (**Figure 5**). To first examine the effects of acute alcohol exposure on the basic parameters of fracture repair, the gross observations of the callus tissue formed in each treatment group at day 14 post-fracture were recorded (**Figure 6**). Using digital calipers to measure external callus diameter, the alcohol-treated group displayed significantly decreased maximum callus diameter as compared to the saline-treated group. Quantitative analysis of newly formed callus tissue utilizing high-resolution micro-computed tomography (μCT) imaging revealed that alcohol-treated mice also display a significant 47% reduction in the volume of callus tissue formed at day 14 post-injury compared to the saline group.

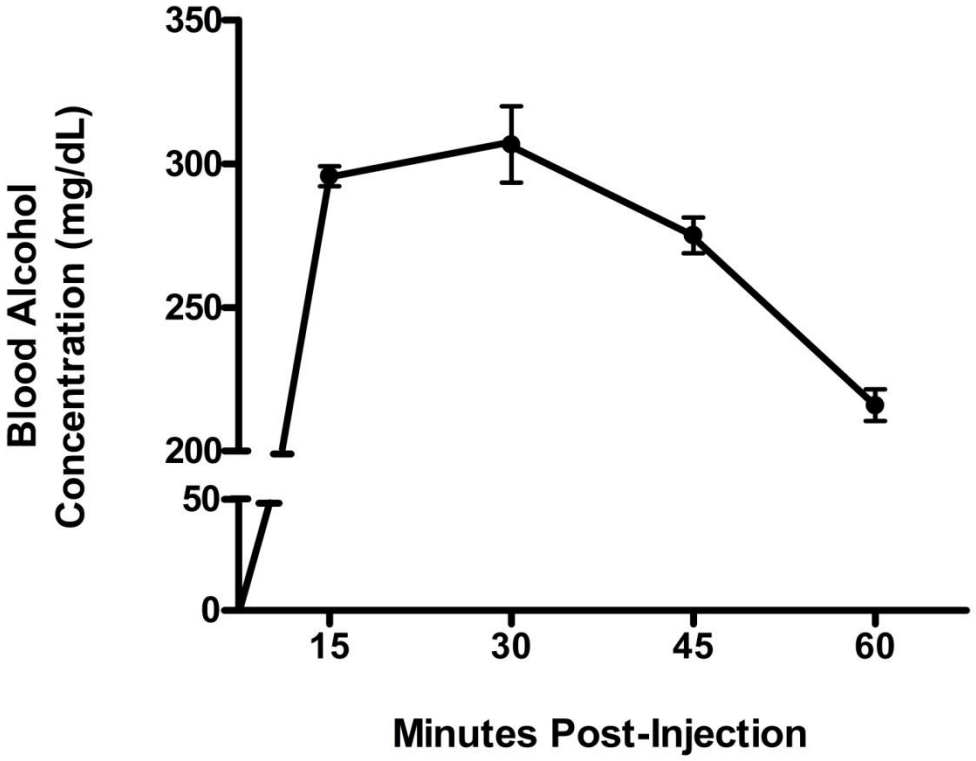

Figure 5. **Blood alcohol concentrations following intraperitoneal injection.** Mice were subjected to 3 consecutive daily doses of a 20% alcohol solution at a dose of 2 g/kg. Mice were sacrificed 15, 30, 45 or 60 minutes post-injection and trunk blood was collected, allowed to clot, and serum samples were tested for blood alcohol using a commercial alcohol reagent kit (Pointe Scientific, Canton, MI). n= 3-5 mice/time point, error bars represent standard deviation of the mean.

DAY 14 POST-FRACTURE	Saline-Treated	Alcohol-Treated
Callus Diameter	3.76 ± 0.42 mm	2.77 ± 0.40 mm *
Callus Volume	3885.62 ± 1126.9 mm^3	2071.40 ± 299.3 mm^3 *

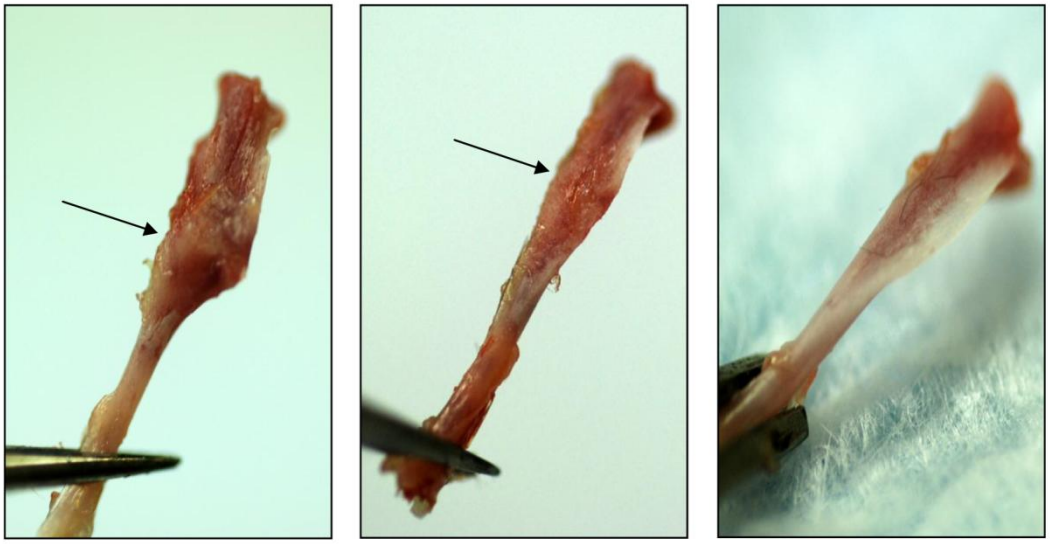

Figure 6. Binge alcohol effects on fracture callus size and volume. Average fracture callus diameter (n=12/group) and volume (n=5/group) are decreased following binge alcohol treatment. Volume was measured using micro-computerized tomography image software. Representative images of post-fracture day 14 calluses from each treatment group. The black arrows denote site of fracture and surrounding callus tissue. *p<0.05, Saline-Treated vs. Alcohol-Treated, Student's *t*-test.

2. Effects of Acute Alcohol on Fracture Callus Tissue Composition

To investigate the effects of alcohol exposure on callus tissue formation and composition histologically, we examined H & E stained sections of fracture callus at 6, 9, and 14 days post-injury. As previously shown in stabilized, mid-diaphyseal mouse tibial fracture models, these time points represent the anabolic phase of external fracture callus formation, in which cartilage formation peaks at day 9, and the overall callus mass is greatest by day 14 (Hiltunen et al., 1993; Le et al. 2001).

As shown in the saline-fracture group in **Figure 7A**, there is abundant cartilage formation in the well-developed external callus (arrow) surrounding the fracture site (indicated by a black line) at post-fracture day 6. By day 9 post-fracture (**Figure 7B**), there is an increase in the amount of cartilage present and in the overall callus size. At this time point, the 3 main compartments of the fracture callus have clearly developed in the saline-treated mice and are delineated in **Figure 8** as an example. The external callus contains abundant hyaline cartilage with hypertrophic chondrocytes and associated zones of endochondral bone formation (dashed line, Figure 8). The periosteal callus appears well-developed and adjacent to the cortices of the bone at the distal end of the fracture site, and the intramedullary (endosteal) callus compartments are clearly defined in the marrow space. By day 14 (**Figure 7C**), the external callus formation has peaked in size, and the cartilaginous matrix of the callus has undergone mineralization, as denoted by the abundant presence of new woven bone. Foci of cartilage remain with associated hypertrophic chondrocytes bordering the ossification center (dashed line, **Figure 9**), revealing that endochondral bone formation is also occurring at day 14 post-fracture.

In contrast to the saline group, callus size does not increase in the alcohol group between days 6 and 9, as seen in **Figure 7D-E**. Minimal cartilage and bone in the external callus has formed through day 14 (arrows). The development of all fracture callus compartments appear to be impacted by alcohol exposure, yet the tissue composition of the external callus appears to be most severely affected. The periosteal callus at day 9 is not clearly defined, and appears to be replaced by immature, myofibroblastic tissue that does not contain developed chondrocytes nor typical cartilage matrix (**Figure 8**). The chondrocytes in the limited amount of hyaline cartilage at day 9 possess an immature and chondroblastic phenotype rather than hypertrophic. In the alcohol group and post-fracture day 14, periosteal bone has appeared adjacent to the fracture site, (**Figure 9**, arrow) however histological evidence of endochondral bone formation is absent, as indicated by the lack of cartilage and hypertrophic chondrocytes in the external callus. Minimal effects on the formation of the endosteal callus at each time point were observed.

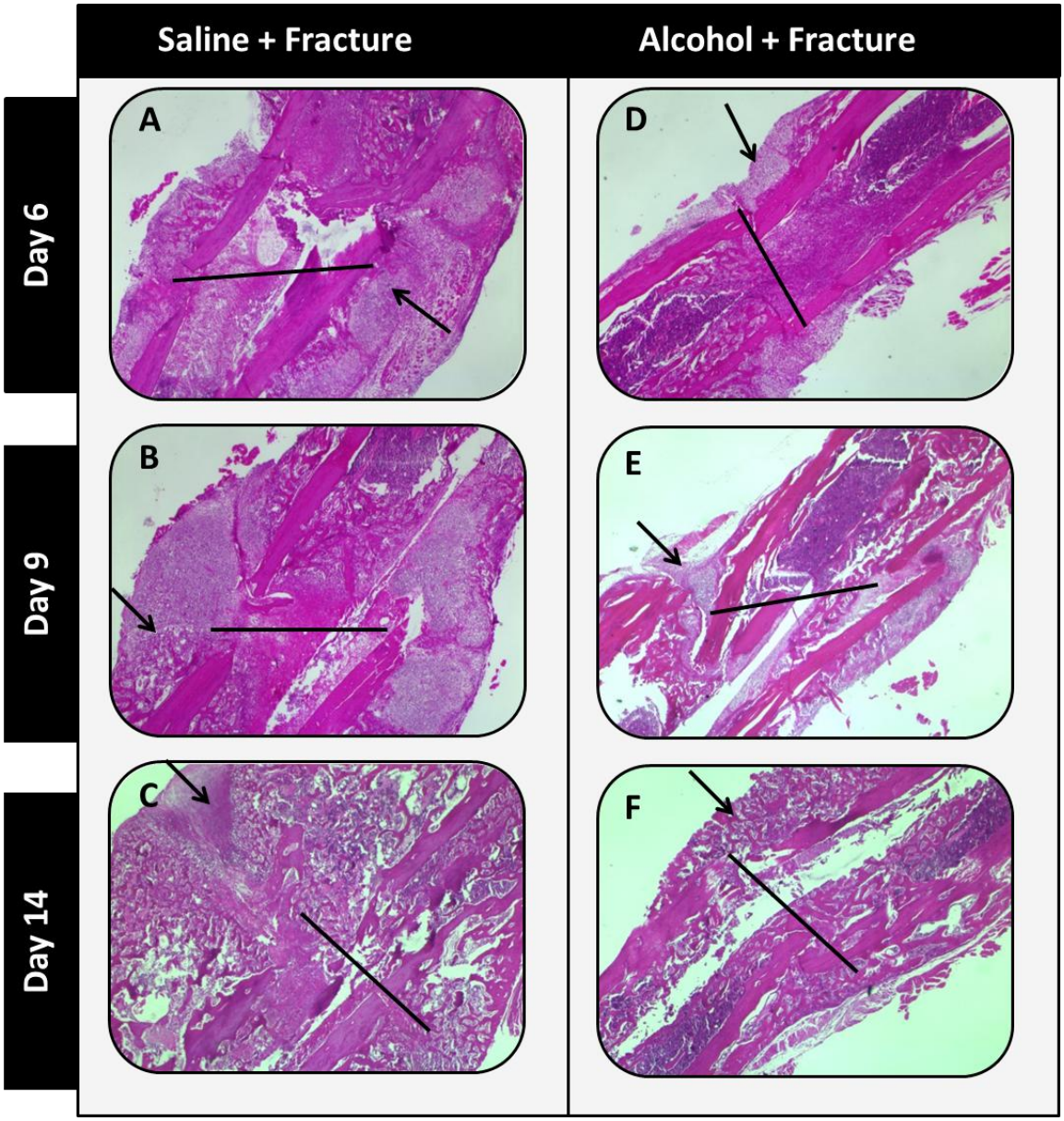

Figure 7. Histological analysis of binge alcohol treatment on fracture callus tissue composition. Representative photomicrographs of H&E stained callus sections from each treatment group at 6, 9, and 14 days post fracture (32x magnification). The black line indicates site of fracture. In the Saline + Fracture group, calluses demonstrateabundant cartilage formation in the external callus (A, arrow) and hypertrophic chondrocytes and endochondral ossification activity (B-C, arrows/inset). In the Alcohol + Fracture group, calluses show a lack of cartilage formation (D-E, arrows), the appearance of immature fibroblastic tissue in place of cartilage (E, inset), and periosteal bone formation rather than endochondral ossification activity (F, arrow).

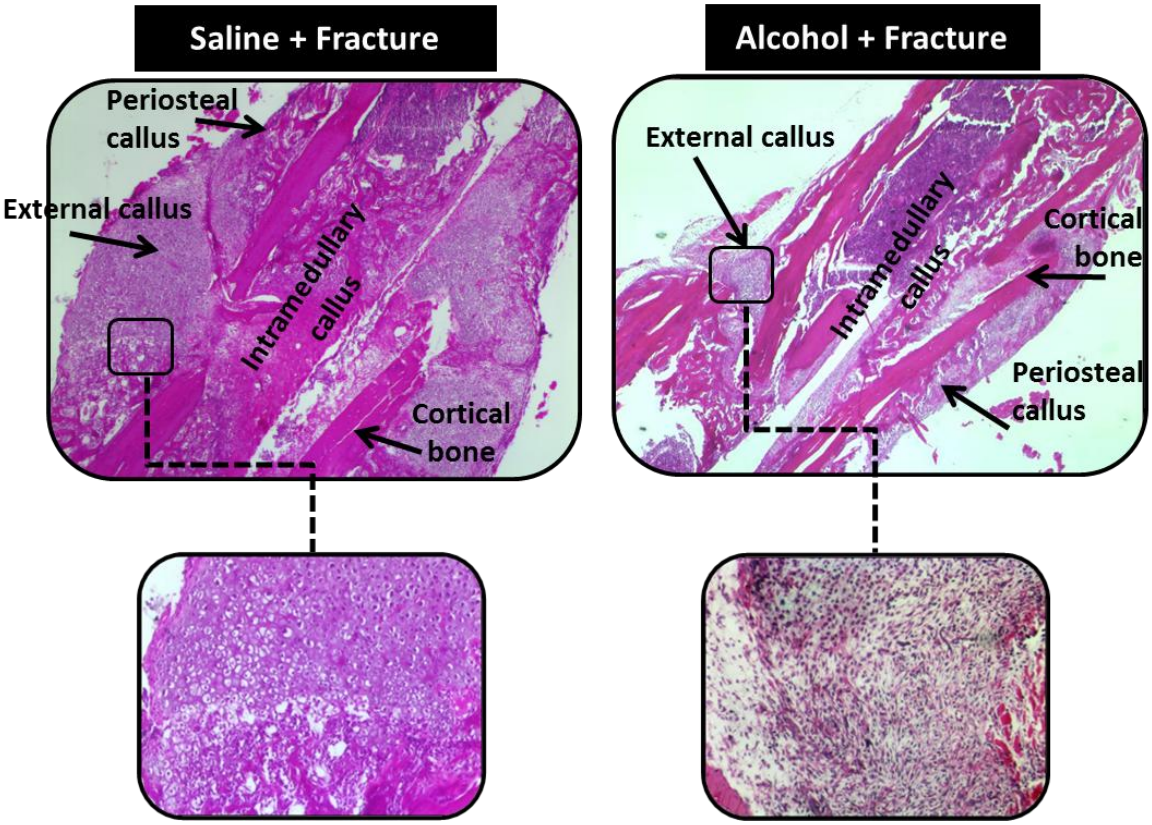

Figure 8: Effects of binge alcohol exposure on fracture callus cartilage formation at day 9 post-injury. Representative photomicrographs (32x magnification) of H & E stained callus sections from day 9 post-fracture demonstrating the effects of alcohol on separate callus components. Binge alcohol inhibits normal formation of the cartilaginous external callus at this time point, and appears to decrease the development of the bony periosteal callus at the distal ends of the fracture site. The bottom images are of higher magnification demonstrating the formation of fibrous, immature tissue in the external callus compartment in place of mature cartilage and areas of endochondral bone formation, as seen in the saline group.

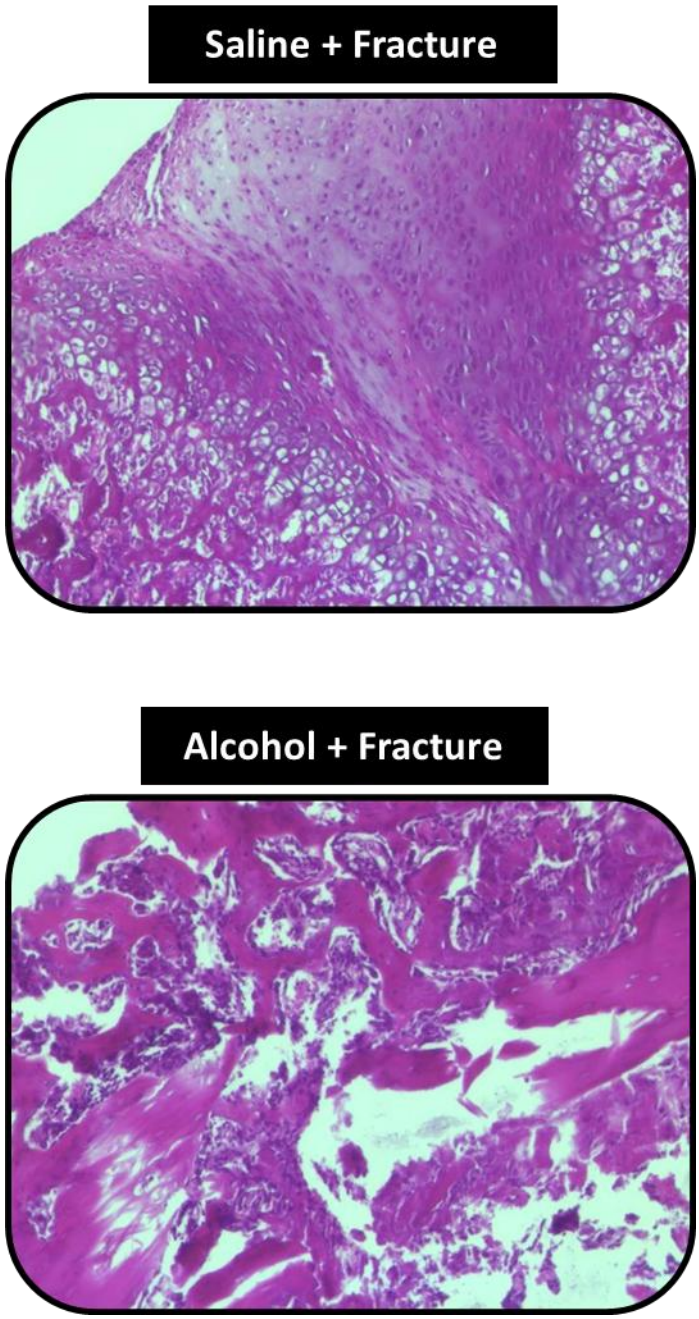

Figure 9: Effects of binge alcohol on endochondral ossification. Representative H&E stained photomicrographs of fracture callus sections demonstrating that binge alcohol exposure prior to fracture inhibits new bone formation via endochondral ossification in the external callus tissue.

3. Effects of Acute Alcohol Exposure on Biomechanical Strength of Fracture Callus Tissue

The four-point bending test is a commonly used method to assess the integrity of fracture healing in long bones (adapted from Hiltunen et al., 1993). Therefore, we tested whether the alcohol-induced changes seen in callus volume and tissue composition result in functional deficits by measuring the biomechanical strength of calluses at day 14 post-fracture. Utilizing a customized four-point bending apparatus, we observed a significant 32% reduction in the maximal load sustained by injured tibias harvested from alcohol-treated mice as compared to tibias from the saline control group (**Figure 10A**). We have previously established that the acute alcohol paradigm utilized in these experiments causes significant bone loss and decreased biomechanical strength of intact rat vertebrae and tibiae (Callaci et al., 2004; Lauing et al., 2008). Therefore, we investigated whether acute alcohol treatment causes bone loss in uninjured mouse tibias by testing the contralateral tibias in the mice from each group, and found there was a significant 17% reduction in maximal load attained in the alcohol-treated group (**Figure 10B**).

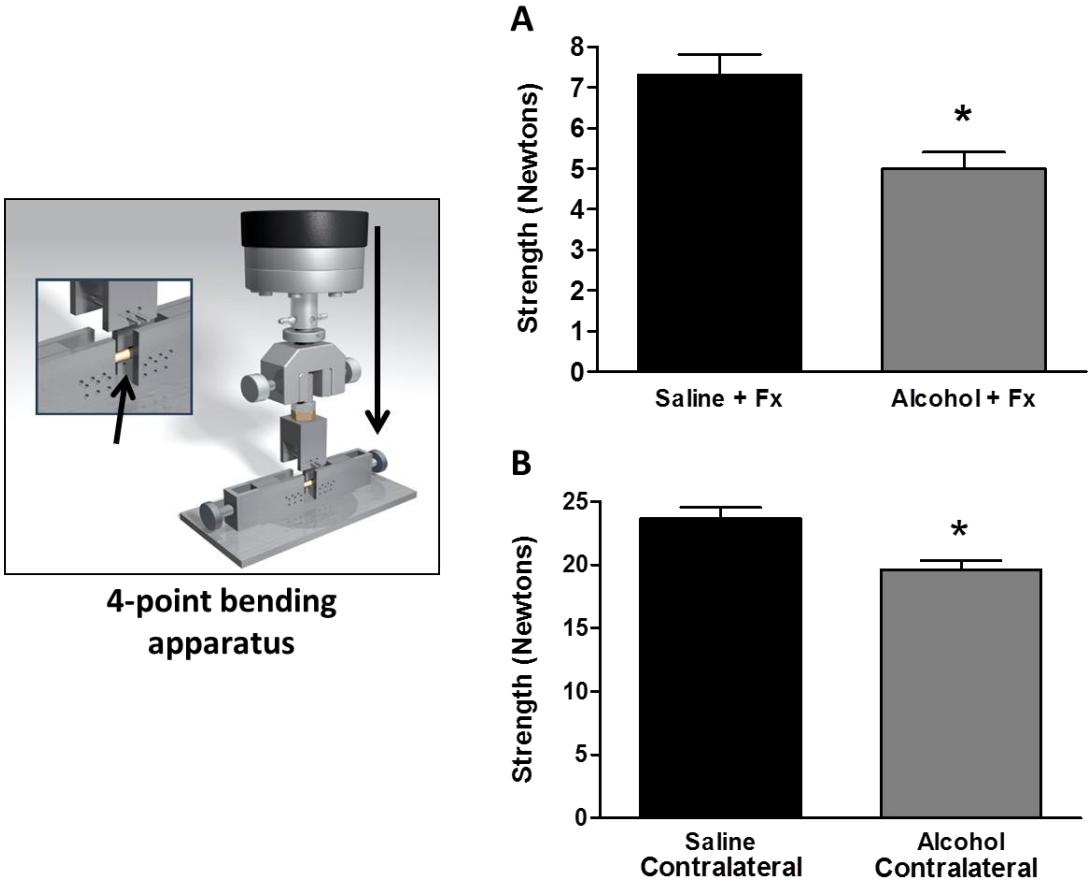

Figure 10. Binge alcohol effects on callus biomechanics 2 weeks post injury. Fracture calluses (A) and contralateral tibias (B) from alcohol-treated mice 14 days post-fracture show significantly reduced bending strength compared to the fracture calluses and contralateral tibias from saline-treated mice. n=14-16/treatment group. *$p<0.05$, Student's *t*-test. Drawing courtesy of Patrick Carrico.

4. Acute Alcohol Effects on β-catenin Expression in the Fracture Callus

Due to the significance of Wnt/β-catenin signaling in normal fracture repair, we investigated whether acute alcohol exposure disrupts the precise regulation of β-catenin levels during fracture healing. Fracture calluses from saline- or alcohol-treated mice were isolated at each time point and total β-catenin protein levels in the fracture callus lysates were examined by western blot. A representative western blot of β-catenin protein expression during fracture repair in saline or alcohol-treated mice is shown in **Figure 11A**. Acute alcohol exposure significantly increased β-catenin protein levels by 2.3-fold at day 6 post-fracture as compared to saline treated controls (**Figure 11B**). At day 9 post-fracture in the alcohol group, the levels of total β-catenin are decreased by 53% compared to levels observed in the saline group (**Figure 11C**). This alcohol-related decrease in β-catenin levels persists until day 14 post-fracture in which the levels of β-catenin are significantly decreased by 56% compared to saline fractured levels (**Figure 11D**). Nearly undetectable β-catenin levels were observed in the contralateral tibias at each time point, with no significant differences noted between alcohol and saline-treated groups.

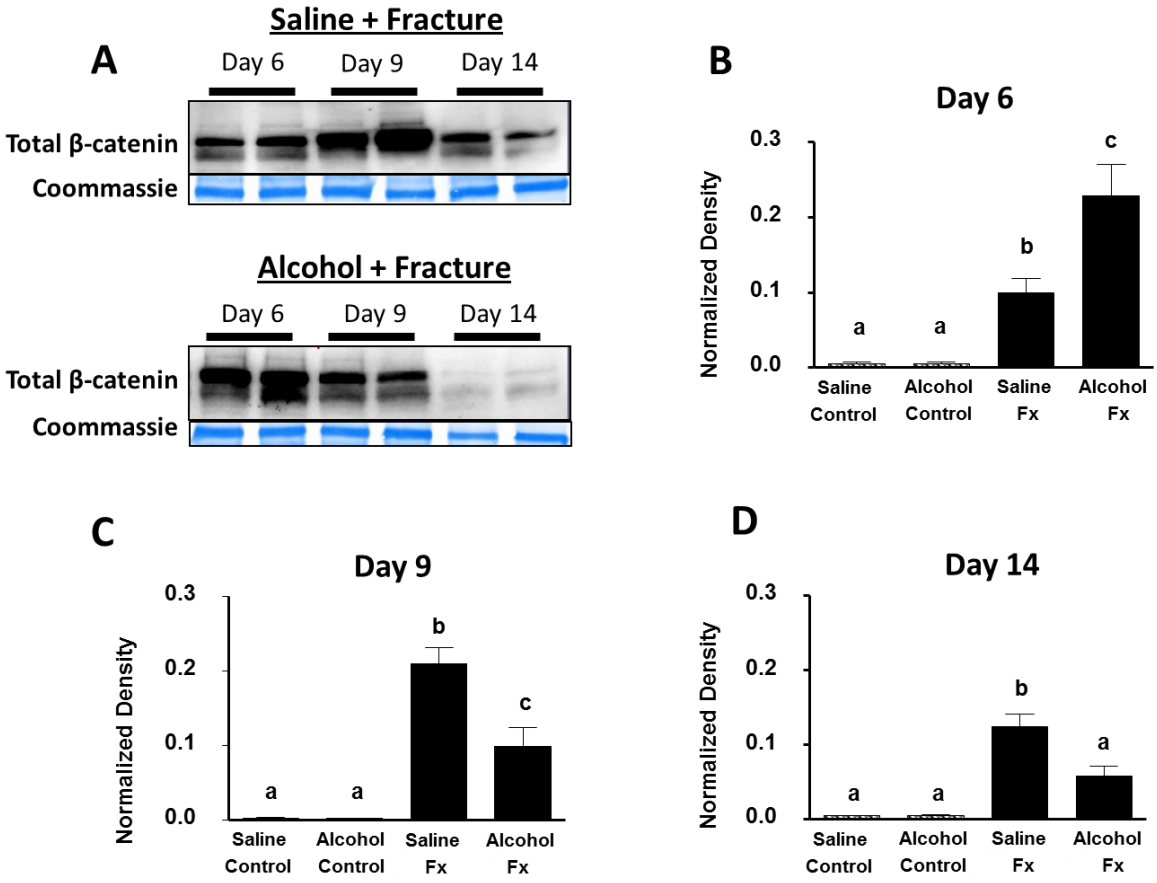

Figure 11. Effects of binge alcohol on total β-catenin levels in the fracture callus. Representative western blot for total β-catenin protein levels in fracture callus lysates at each timepoint. To ensure equal loading, blots were Coomassie-stained after β-catenin detection and normalized to a 60 kD band. Data are presented as the densitometric ratio of total β-catenin/Commassie-stained band. Alcohol causes a significant increase in total β-catenin protein levels at day 6 (B), followed by a marked reduction in β-catenin levels at days 9 and 14 post-fracture (C-D). Groups not sharing a letter are significant, $p \leq 0.05$ using one-way ANOVA and Tukey's multiple comparison procedure.
n = 4-8 mice/treatment group

5. **Effects of Acute Binge Alcohol on Wnt-Specific Transcriptional Activation in the Fracture Callus**

Since β-catenin serves as the transcriptional coactivator of the canonical Wnt pathway, we sought to determine whether alcohol-induced modulation of β-catenin levels subsequently leads to changes in canonical Wnt transcriptional activation. In order to test this, we examined histological sections of fracture callus from TCF-transgenic mice (Jackson Labs, Bar Harbor, ME) that express β-galactosidase in the presence of β-catenin/TCF-driven transcription, which occurs predominantly through Wnt stimulation. **Figure 12A-C** demonstrates the temporal changes in Wnt transcription throughout healing in the saline-treated group. At day 6, positive *LacZ* staining is present within the cartilage most distal to the fracture site, consistent with activated β-catenin/TCF transcription within pre-hypertrophic chondrocytes and immature osteoblasts (arrow). At day 9 post-fracture, the *LacZ* staining in the saline group is robust in the trabeculae of new woven bone and in areas surrounding the hypertrophic cartilage (arrow), indicative of actively mineralizing osteoblasts replacing the cartilaginous matrix with bone. By day 14, *lacZ* staining is still abundant in the periosteal callus and in the bony callus tissue being formed furthest from the fracture site (arrows)**.**

As seen in **Figure 12D-F,** there are differences in the spatial orientation and intensity of *LacZ* staining in the alcohol group as compared to saline control calluses. At day 6 in the alcohol-treated group, strong *LacZ* staining appears mainly concentrated in the marrow cavity adjacent to the fracture site (arrows), with a small amount of staining present in the myofibroblastic, undifferentiated tissue of the external callus. By day 9,

there is a significant decrease in *Lacz* staining intensity (arrow) and cartilage formation. Similarly, the positive staining at day 14-post fracture in the alcohol group is almost entirely absent with the exception of a few foci in the most peripheral bone tissue in the external callus (arrow).

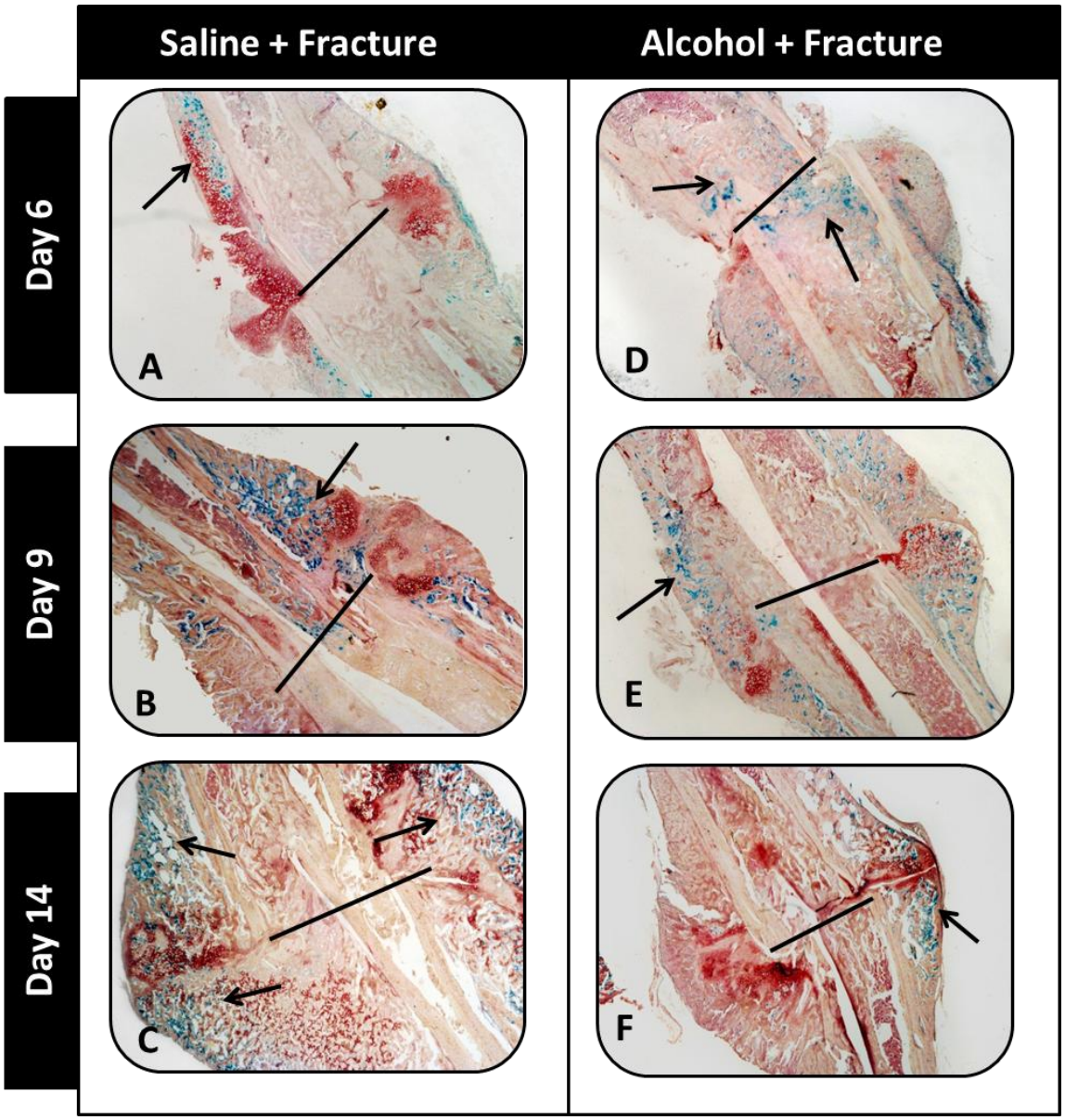

Figure 12. Binge alcohol effects on Wnt-specific transcriptional activation in the fracture callus. Representative callus sections counterstained with neutral red from TCF-transgenic mice expressing lacZ are shown for each time point (32x magnification). Arrows in the Saline + Fracture group (A-C) denote abundant Wnt transcriptional activation in areas of active endochondral ossification. Arrows in post-fracture day 6 in the Alcohol + Fracture group (D) show a concentration of Wnt transcriptional activation in the medullary canal. Days 9-14 post-fracture show a reduction in the overall intensity of positive lacZ staining in the callus tissue compared to saline-treated mice (E-F, arrows).

DISCUSSION

In this study, we show that acute alcohol exposure has a deleterious effect on fracture repair in mice as demonstrated by the reduction in callus size, biomechanical strength, and alteration in callus tissue composition at the injury site. These effects persist up to 14 days post-fracture. These findings parallel a concomitant deregulation of protein levels of a key canonical Wnt signaling molecule, β-catenin, as well as β-catenin/T-cell factor (TCF) transcriptional activation. Numerous clinical studies have associated alcohol abuse with an increased risk of sustaining an orthopaedic injury and with decreased healing potential (Foulk and Szabo, 1995; Perlman and Thordarson, 1999; Mathog et al., 2000; Williams et al., 2008; Duckworth et al., 2011), and many animal studies have demonstrated alcohol-induced bone damage and impaired healing utilizing a chronic exposure model (Jänicke-Lorenz and Lorenz, 1984; Chakkalakal et al., 2005; Trevisiol et al., 2007). However, few studies to date have investigated a molecular target of alcohol during repair, and more specifically, following acute alcohol exposure. The identification of β-catenin signaling and Wnt transcriptional activation in this study as significant targets of alcohol exposure in healing bone tissue supports our previous findings in uninjured bone, in which over 40 genes belonging to the canonical Wnt pathway were modulated following acute alcohol exposure (Himes et al., 2008).

Our gross observations and histological data show that alcohol exposure is most inhibitory to the formation of the external callus tissue and the endochondral ossification process. Not surprisingly, the alcohol-related decrease in callus tissue formation resulted

in a reduction in load-bearing properties of day 14 fracture calluses as demonstrated by the biomechanical 4-point bending test. Acute alcohol exposure had a striking effect on cartilage formation compared to the saline groups at days 6 and 9 post-fracture, and resulted in the appearance of immature fibrous tissue rather than mature, hypertrophic cartilage. These observations parallel similar effects seen on external callus cartilage formation utilizing a rat model of acute binge alcohol exposure and femoral fracture repair established in our laboratory (Volkmer et al. 2011). Though we expected to see an overall decrease in bone and cartilage due to the importance of Wnt/β-catenin signaling in osteoblast and chondrocyte formation, these results were particularly remarkable because the duration of alcohol exposure in this study was much shorter compared to previous studies reporting similar effects on external callus formation and fibrous tissue proliferation following chronic alcohol exposure (Elmali et al., 2002; Chakkalakal et al., 2005; Trevisiol et al., 2007).

Some bone and cartilage formation does occur in the alcohol group, indicating that this model of acute alcohol exposure does not completely inhibit fracture repair. We do not have functional or biological data extending further than 14 days post-injury, and though we speculate that while this brief alcohol exposure prior to injury does not cause permanent healing defects, it is sufficient to delay the crucial and tightly regulated early phases of fracture repair. In addition, the acute alcohol exposure utilized in this study does not fully represent a clinically prevalent situation in which injured patients presenting with acute alcohol intoxication are likely not first-time drinkers, nor are likely to cease drinking during the healing process. Nonetheless, our data indicate that a short

exposure to high levels of alcohol prior to orthopaedic injury has the capacity to initiate a cascade of cellular events that lead to fracture repair deficiencies up to 2 weeks post-injury.

The formation of the external callus is heavily dependent upon the recruitment and differentiation of mesenchymal stem cells to the fracture site (Devine et al., 2002; Shen et al., 2002; Taguchi et al., 2005; Kumagai et al., 2008). A key regulatory pathway involved in mesenchymal stem cell differentiation into cartilage and bone is the Wnt/β-catenin pathway. The precise regulation of β-catenin levels during early fracture repair has been shown to be essential for fracture union by controlling cartilage and bone formation within the external callus tissue (Chen et al., 2007; Huang et al., 2011). Deletion of β-catenin only in committed osteoblasts during fracture repair inhibits external callus mineralization, indicating that the osteoblast-specific regulation of β-catenin is required for fracture union and callus bone formation (Chen et al., 2007). The controlled timing of Wnt/β-catenin signaling activation is also required for endochondral ossification and chondrocyte maturation, and deletion of Wnt pathway proteins or ectopic expression of Wnt/β-catenin signaling in chondrocytes inhibits these processes (Tamamura et al., 2005; Zhong et al., 2006; Chen et al., 2008).

In our acute alcohol exposure model, we observe a 2.3-fold increase in β-catenin levels in the alcohol group at day 6 compared to the saline group, while the levels at day 9 and 14 in the alcohol group are 53% and 56% lower, respectively. Peak β-catenin levels in the saline group are observed at day 9 post-fracture, when endochondral ossification has initiated, osteoblasts are actively recruited, and chondrocyte maturation

and hypertrophy is evident. Alcohol exposure prior to injury shifts the peak expression of β-catenin to an earlier time point, day 6, which is accompanied by prolonged decreases in cartilage formation with minimal chondrocyte hypertrophy and the appearance of highly cellular, immature fibrous tissue in place of cartilage. These changes persist until day 14, where we observe no evidence of endochondral bone formation. This suggests that one possible mechanism underlying these observations is that the alcohol-related deregulation of β-catenin levels occurs at a critical developmental stage of fracture healing and external callus formation. Aberrant activation of Wnt/β-catenin signaling is known to be involved in the development of fibrosis (reviewed in Lam and Gottardi, 2011), and may link our observations of the development of nonspecific fibrous tissue in the callus at day 9 in the alcohol group with deregulation of β-catenin levels. As mentioned above, previous reports have established the requirement of Wnt/β-catenin signaling during chondrocyte maturation and subsequent endochondral ossification during development and repair (Kitagaki et al., 2003; Tamamura et al., 2005; Chen et al. 2008; Huang et al., 2011), which support that the alcohol-related inhibition of endochondral bone formation process at these time points may be directly attributed to the deregulation of β-catenin levels.

The TCF-transgenic mouse model provides further evidence that the canonical Wnt pathway is a significant target of alcohol in healing bone. This mouse model allows the expression of β-galactosidase in the presence of activated T-cell factor/Lymphoid enhancer factor (TCF/LEF) transcription factors by β-catenin binding, which is a necessary event of canonical Wnt pathway activation. Although not completely

exclusive to canonical Wnt transcriptional activaton, this mouse model is widely used to investigate the involvement of canonical Wnt signaling in various tissues. Chen et al. (2007) has previously characterized the expression of Wnt-specific transcriptional activation during normal fracture repair in the mouse, and similarly to their observations, we detect strong *LacZ* staining in the saline group at days 6 and 9 in areas consistent with the maturation of osteoblast precursors and hypertrophic chondrocytes. This is associated with peak β-catenin expression during the anabolic phase of external callus formation.

In contrast, the alcohol group displays striking spatial and quantitative changes in Wnt transcriptional activity within the fracture callus. Theoretically, changes in β-catenin protein expression, if relevant to the canonical Wnt pathway, should lead to changes in β-catenin/TCF-mediated transcriptional activity. At day 6 post-fracture in the alcohol group, *Lacz* staining is strongest in the bone marrow near the fracture site rather than in the external callus tissue, which correlates to the significant increase in β-catenin protein levels at this time point compared to saline-treated controls. This Wnt transcriptional deregulation in the bone marrow provides further evidence that a deregulation of Wnt/β-catenin signaling may contribute to the appearance of immature, fibrous tissue by day 9 post-fracture instead of normal hyaline cartilage. Ultimately, these experiments demonstrate that in addition to deregulating β-catenin protein levels, acute alcohol exposure subsequently caused deregulation of transcriptional activation of the Wnt pathway during critical stages of fracture callus formation, which can then lead to alterations in the expression of Wnt target genes that are necessary for fracture repair.

We cannot rule out the possibility that other pathways may be activating β-catenin. Some studies have shown that β-catenin can also be activated and translocated to the nucleus in a Wnt-independent manner following stimulation of growth factor receptors and tyrosine kinase activation (Haraguchi et al. 2004, Ji et al. 2009, Schramp et al. 2011). Others have observed that osteoblasts can form from mesenchymal stem cells via β-catenin/TCF interaction, yet this interaction is not stimulated by Wnt signaling (Qiang et al., 2009). These data provide an alternative mechanism for Wnt-independent β-catenin activation, however, previous fracture models utilizing various *in vivo* methods such as antibody-mediated inhibition of endogenous canonical Wnt antagonists, knockout of the essential Wnt coreceptor Lrp5 , or utilizing lithium chloride to inhibit GSK-3β have supported that Wnt/β-catenin signaling predominates over other pathways in bone during fracture repair and is a central regulator of bone healing (Chen et al., 2007; Gaur et al., 2009; Komatsu et al., 2010; Ominsky et al., 2010). Lastly, in order to completely assess the effects of alcohol on activated β-catenin, nuclear levels must be specifically measured; however the Wnt-related transcriptional activation data provided by the TCF-transgenic mice support our observations that acute alcohol exposure can disrupt nuclear β-catenin/TCF signaling activity.

In summary, these data provide valuable insight into the molecular effects of alcohol during bone repair, and allow further research to be conducted on therapies to prevent or reverse alcohol-induced deregulation of Wnt/β-catenin signaling in alcohol-abusing patients sustaining an orthopaedic injury.

CHAPTER IV

THE EFFECTS OF ACUTE BINGE ALCOHOL EXPOSURE ON FRACTURE HEALING ARE ATTENUATED BY LITHIUM CHLORIDE TREATMENT

ABSTRACT

Background: Binge alcohol exposure in mice is associated with decreased fracture callus size and strength and a marked reduction in the presence of hyaline cartilage and bone tissue at the site of injury. Endochondral ossification, the most common mode of bone formation during fracture healing, is inhibited by alcohol exposure. The Wnt pathway, through tight regulation of stabilized β-catenin levels, plays a pivotal role in the process of endochondral ossification, cartilage maturation, and osteoblast differentiation. For the current study, we sought to determine whether the alcohol-induced damage on fracture repair could be reversed by exogenously enhancing stabilized β-catenin levels in alcohol-treated mice utilizing lithium chloride (LiCl).

Methods: C57BL/6 or TCF-transgenic mice were exposed to intraperitoneal alcohol (2g/kg) or saline for 3 consecutive days. One hour after the third injection, mice received a stabilized tibial fracture and were sacrificed 6, 9, or 14 days post-injury. Two groups of mice also received daily injections of 100 mg/kg LiCl subcutaneously beginning 4 days post-injury. Relative levels of "activated" versus "inactivated" levels of β-catenin and GSK-3β in fracture calluses were measured by western blot utilizing antibodies against

specific phosphorylation sites on the proteins.

Results: Average serum lithium levels were not different between treatment groups. As demonstrated previously, alcohol was associated with decreased callus size, biomechanical strength, suppression of endochondral ossification, and deregulation of β-catenin levels. In fracture callus lysates, western blot revealed that alcohol exposure decreased the level of active, hypophosphorylated β-catenin and increased the level of inactive, hyperphosphorylated β-catenin. This was paralleled by an increase in activated GSK-3β. LiCl treatment partially reversed these effects by augmenting stabilized β-catenin protein levels and decreasing the amount of activated GSK-3β back to control levels, restoring endochondral ossification and cartilage formation, and increasing callus strength in alcohol-treated mice.

Discussion: These data show a link between alcohol exposure, impaired fracture healing, and deregulation of Wnt signaling. We show that exogenous activation of the Wnt pathway during healing may improve fracture repair in patients at risk for alcohol-related complications.

INTRODUCTION

Canonical Wnt signaling during fracture repair plays a pivotal role in promoting the formation of osteoblasts and chondrocytes, the main cell types involved in creating the fracture callus during the early healing phases. Its precise regulation has been shown to be essential in a variety of tissues in order to maintain the course of proper wound healing, including bone fracture (Chen et al. 2007, Kakar et al. 2007, Huang et al. 2012). Signaling through the canonical Wnt pathway regulates both embryonic development and adult homeostasis in multiple organ systems thus the pathway is tightly regulated to prevent excessive stimulation and maintain cellular homeostasis.

Aberrant canonical Wnt signaling and abnormal β-catenin stabilization are responsible for multiple pathological states (Fodde and Brabletz, 2007, Varallo et al. 2007, Stojadinovic et al. 2005, Caricasole et al. 2005), highlighting the potentially adverse effects of Wnt signaling deregulation. In bone tissue, canonical Wnt signaling has recently emerged as a central regulator of bone homeostasis and fracture repair. Point mutations in the Wnt co-receptor Lrp5, resulting in gain- or loss-of-function of this receptor, are directly responsible for two rare bone mass disorders: high bone mass phenotype (HBM) and osteoporosis-pseudoglioma syndrome (OPPG) (Gong et al. 2001, Boyden et al. 2002). Of particular significance to the research performed in our laboratory, recent studies have demonstrated that the precise regulation of Wnt/β-catenin signaling during fracture repair is required to achieve normal healing (Chen et al. 2007, Huang et al. 2012). The main effector of the pathway, β-catenin, plays an essential role in canonical Wnt signaling, as it is the transcriptional co-activator of the TCF/LEF family of

proteins in the nucleus (Behrens et al. 1996, Molenaar et al. 1996). In bone cells, the proportion of β-catenin involved in nuclear transactivation is low compared to levels associated with membrane-bound cadherins. Therefore, given the significance of aberrant β-catenin signaling in disease and more specifically, during bone healing, this small pool of activated β-catenin must be tightly regulated due to its potent signaling capabilities.

The stability, function, and cellular location of β-catenin are controlled by post-translational modifications including phosphorylation and ubiquitination. The essential negative regulator of the Wnt/β-catenin pathway is GSK-3β, is a serine/threonine kinase that when activated, hyper-phosphorylates β-catenin to target it for degradation (Yost et al. 1996). Following a "priming" phosphorylation by CK1 at serine 45 (S45), GSK-3β sequentially phosphorylates the amino-terminus of β-catenin in the order of threonine 41 (T41), serine 37 (S37) and serine 33 (S33), targeting β-catenin for ubiquitination and degradation by the proteasome (Liu et al. 2002). When β-catenin is unphosphorylated at these sites, it is thought to be "activated", or available for nuclear signaling, resulting in TCF/LEF-mediated transcriptional activation to stimulate Wnt-mediated target gene expression (Staal et al. 2002).

The canonical Wnt pathway can be indirectly stimulated by using inhibitors specific for GSK-3β, which results in increased stabilization of β-catenin. Lithium salts, including LiCl, are effective in preventing GSK-3β-mediated hyperphosphorylation of β-catenin, therefore increasing canonical Wnt signaling. Studies in bone tissue, as well as other organ systems, commonly utilize lithium as an inhibitor of GSK-3β to increase

canonical Wnt signaling both *in vivo* and *in vitro* (Clement-Lacroix et al. 2005, Chen et al. 2007, Nakanishi et al. 2008,). In a clinical setting, lithium is used as a psychotropic drug. Coincidentally, patients treated with lithium for long periods of time have an associated decreased risk of fracture and increased bone mass (Vestergaard 2008, Zamani et al. 2008). Chen et al. (2007) have demonstrated that the utilization of LiCl treatments to augment the canonical Wnt pathway during fracture repair must be precisely timed to result in improved fracture repair, suggesting that increasing stabilized, active β-catenin levels too early in the repair process can delay or inhibit normal healing.

In the previous chapter, we show that acute alcohol exposure modulates total β-catenin levels throughout the critical stages of fracture callus formation and is associated with decreased callus strength, impaired bone and cartilage formation, and modulation of β-catenin/TCF-dependent transcription. In the following experiments, we use a similar binge alcohol-tibial fracture model in which mice receive 2 weeks of binge alcohol exposure followed by tibial fracture. To test whether alcohol-induced deficient fracture repair is linked to deregulation of β-catenin signaling, we administered daily LiCl to mice beginning at 4 days post-fracture and observed whether exogenous enhancement of the canonical Wnt pathway could restore normal bone healing in alcohol-exposed mice. We hypothesized that inhibiting GSK-3β activity with LiCl treatment would restore biomechanical strength, increase bone and cartilage formation in the fracture callus and increase stabilized β-catenin levels in binge alcohol-treated mice.

RESULTS

1. Fracture Callus Tissue Composition and Endochondral Ossification in Binge Alcohol-Treated Mice Following Lithium Chloride Treatment

Our laboratory previously reported that fracture calluses from binge alcohol-treated mice throughout day 14 of healing show a significant decrease in callus size, cartilage and bone formation and inhibition of endochondral ossification (Lauing et al. 2012). Based on these studies, we first examined the effects of lithium chloride (LiCl) treatment on fracture callus tissue composition in alcohol-exposed mice. The mice received daily subcutaneous (s.c.) injections of LiCl beginning at day 4 post-fracture, a time point that has previously been reported to improve the fracture repair process (Chen et al. 2007). No significant differences in serum lithium levels were noted between the alcohol and saline groups and the levels of lithium were within the reported therapeutic target range in humans of 0.5-1.5 mmol/L (**Figure 13**).

In callus tissue from vehicle-treated saline mice, there is abundant mature hyaline cartilage in the external callus at day 14 post-fracture (**Figure 14A**). Consistent with our previous observations at this time point (Lauing et al. 2012), an ossification zone is evident (**Figure 15A, dashed line**) with associated hypertrophic chondrocytes (arrow), supporting that stabilized tibial fracture repair in saline-treated mice occurs predominantly through endochondral bone formation. In contrast, the alcohol group demonstrates little hyaline cartilage formation or associated endochondral bone formation activity in the external callus at day 14 post-fracture (**Figure 14B).** Also consistent with

our previous findings, the fracture callus tissue present in the alcohol-treated mice appears of periosteal origin (**Figure 15B)**.

In the group receiving saline and LiCl, there are no significant effects of LiCl treatment on cartilage development, endochondral bone formation (**Figure 14C**), or chondrocyte hypertrophy (arrow). However, LiCl treatment markedly improved theabnormal callus tissue composition observed in alcohol-treated mice, as demonstrated in **Figure 14D**. The appearance of mature cartilage tissue, along with associated zones of ossification (**Figure 15D,** dashed line) and hypertrophic chondrocytes (arrow), indicate that LiCl treatment restores normal callus tissue composition by promoting endochondral bone formation in binge alcohol-treated mice.

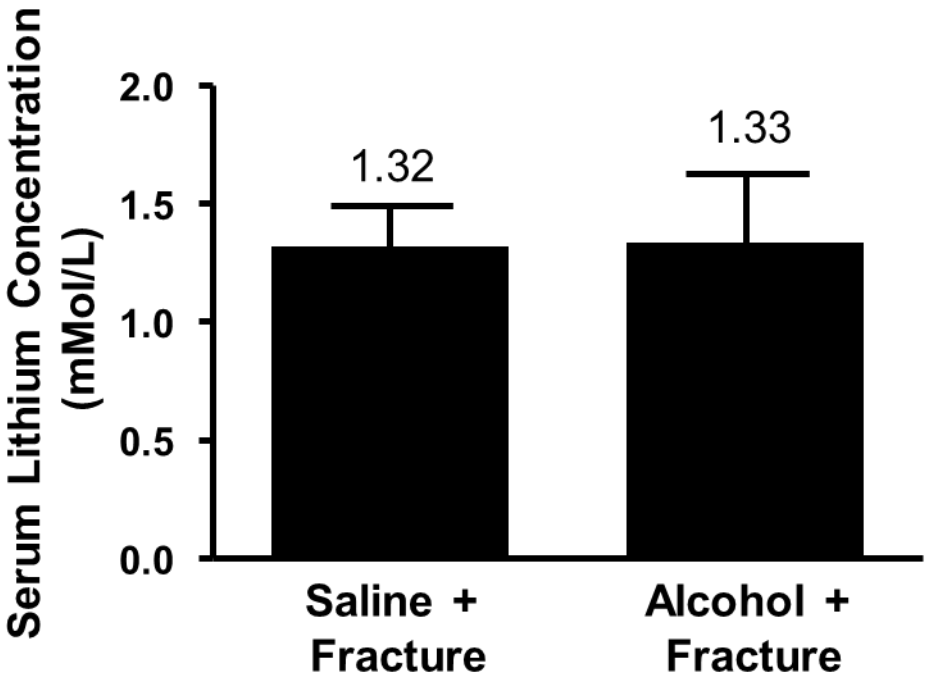

Figure 13. Serum lithium concentrations following subcutaneous injection of lithium chloride in mice. Mice were administered daily subcutaneous (s.c.) injections of LiCl on days 4 through 14 post-fracture. Three hours after the final injection, blood was collected and serum lithium levels were measured. No significant differences are noted between treatment groups. n=5-6 mice/treatment group.

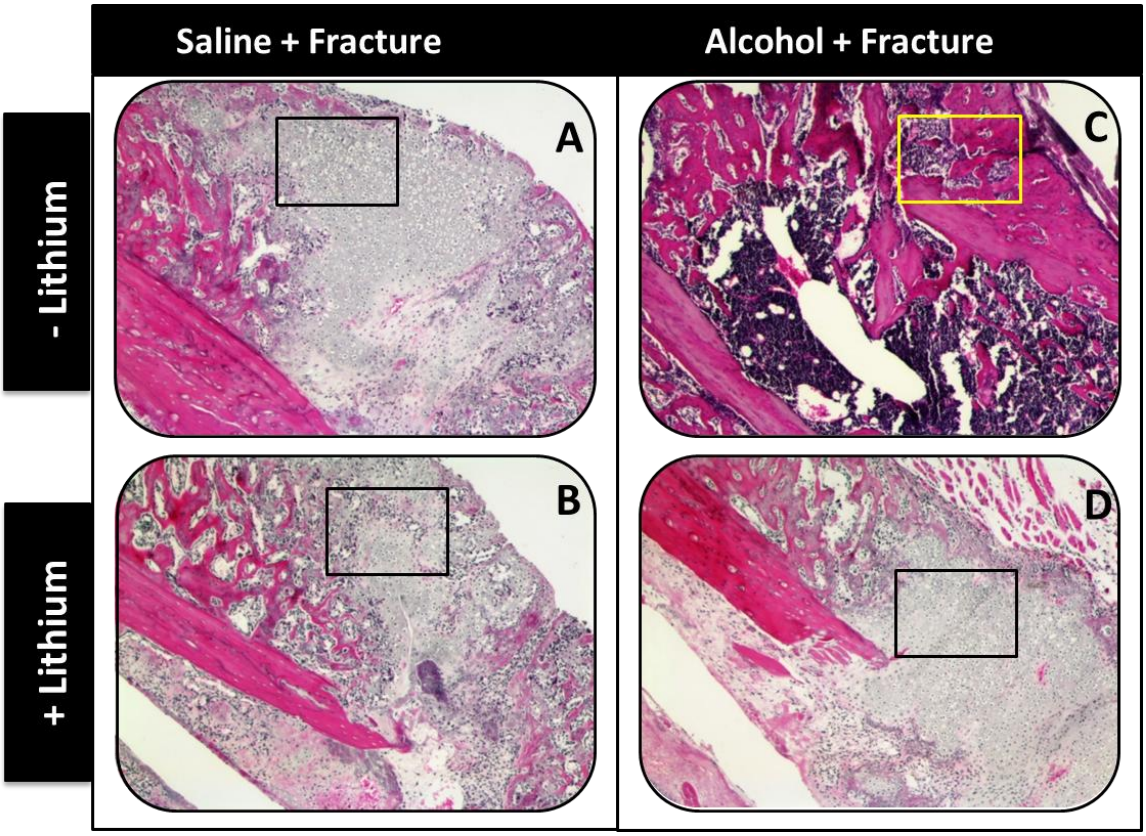

Figure 14. **Histological effects of LiCl treatment in binge-alcohol exposed mice 14 days post-fracture.** Representative photomicrographs of H & E stained callus sections at day 14 post-fracture (50x magnification) show that 2 weeks of binge alcohol exposure inhibited endochondral ossification and cartilage formation (B). Following LiCl treatment, the alcohol-exposed mice demonstrate normal, mature cartilage and restoration of endochondral ossification (D). Higher magnification of the boxed areas are seen in **Figure 15** to highlight the effects of LiCl treatment on endochondral bone formation.

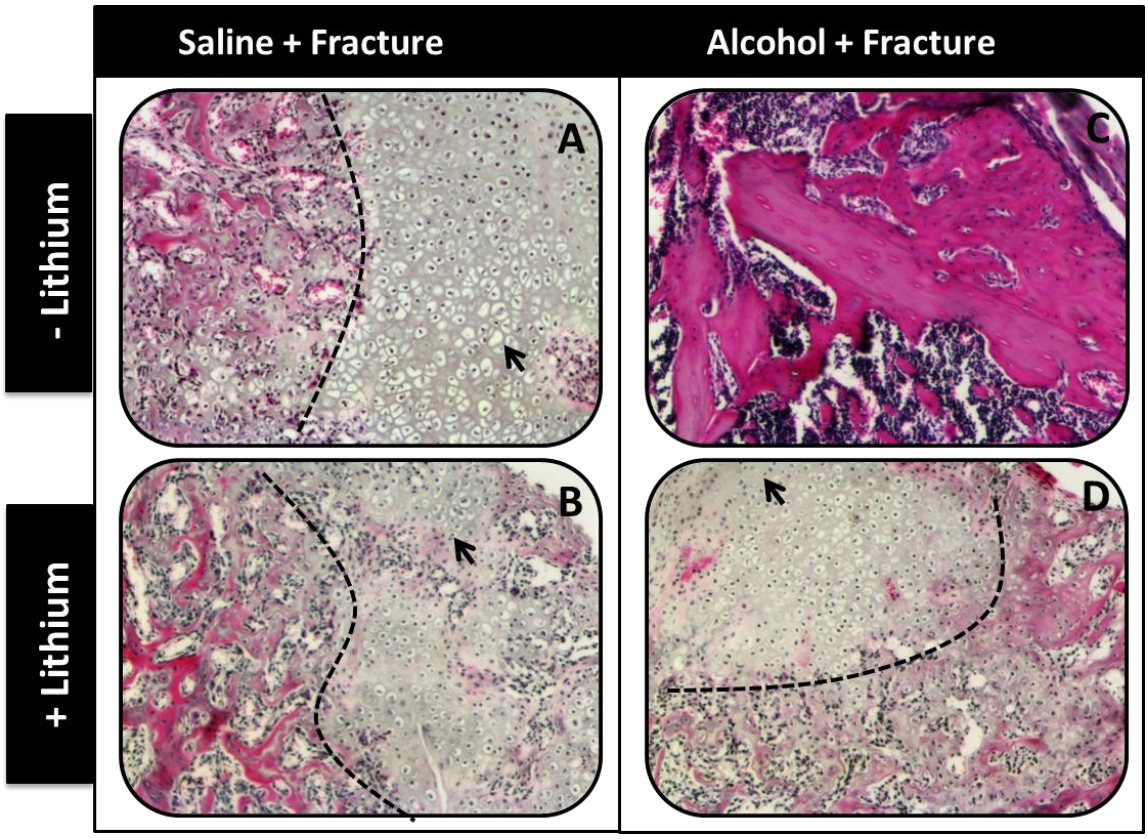

Figure 15: Effects of LiCl on endochondral bone formation in alcohol-exposed mice. Images are 100x magnification of the above images. The dashed lines indicate the endochondral zones of ossification. Arrows indicate enlarged, mature chondrocytes in the zone of hypertrophy. Mice treated with alcohol alone show no evidence of endochondral ossification or mature hypertrophic chondrocytes (B). LiCl treatment reversed the histological phenotype of binge alcohol-treated mice and caused mature cartilage formation with associated hypertrophic chondrocytes near the zone of ossification (D). No significant changes caused by LiCl treatment were noted in the saline group (C).

2. Effects of Lithium Chloride Treatment on Biomechanical Strength of Fracture Callus Tissue in Binge Alcohol-Exposed Mice

In the previous chapter, we observed a significant decrease in the biomechanical strength of fracture callus tissue from alcohol-treated mice at day 14 post-injury. To examine whether LiCl treatment following binge alcohol exposure improved fracture callus biomechanical parameters, we utilized the identical four-point bending apparatus and load-to-failure method as previously described (Hiltunen et al. 1993) to test the maximum load capacity of injured and contralateral tibias harvested at day 14 post-fracture (**Figure 16A**). Fracture calluses from alcohol-exposed mice show a significant 32% reduction in biomechanical strength. With the addition of LiCl, the biomechanical strength of alcohol-treated mice improved to 88% of the strength observed in saline-treated mice receiving LiCl. Lithium treatment had no effect on the strength of the fracture callus tissue between saline-treated groups.

In contralateral tibias, alcohol exposure significantly reduced the four-point bending strength by 17% as compared to saline-treated control uninjured tibias. Following LiCl treatment, the biomechanical strength of the contralateral tibias of binge alcohol-exposed mice was significantly increased by 14% compared to mice receiving binge alcohol treatment alone, while no change was observed in the saline-treated groups. **Figure 16B**).

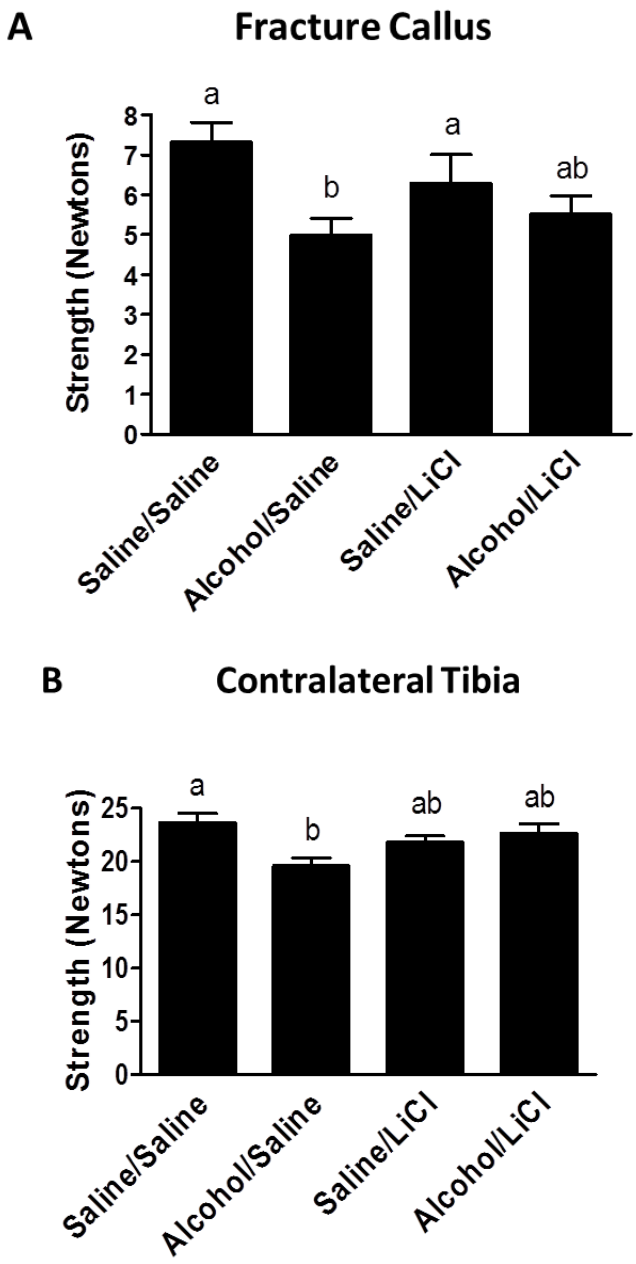

Figure 16: Effects of LiCl on biomechanical strength of the fracture callus following binge alcohol exposure. The maximum load sustained of fracture calluses (A) and contralateral tibias (B) from either alcohol or saline-exposed mice, with or without LiCl treatment at 14 days post-fracture. Alcohol exposure significantly reduced bending strength of fracture calluses and contralateral tibias compared to saline-exposed mice. LiCl partially restored fracture callus strength and significantly increased contralateral tibia strength in alcohol-exposed mice. Groups not sharing a letter are significant, $p \leq 0.05$ using one-way ANOVA and Tukey's multiple comparison procedure. n = 14-18 mice/treatment group

3. Effects of Acute Binge Alcohol Exposure and Lithium Chloride Treatment on Active and Inactive β-catenin Protein Levels

Next, we wanted to examine whether the significant alcohol-related decrease in total β-catenin levels observed in the previous chapter corresponds to a decrease in active, hypophosphorylated β-catenin following binge alcohol exposure. Therefore, we measured levels of the hypophosphorylated (active) form of β-catenin, which is unphosphorylated at the N-terminal residues S33/S37/T41, as well as the hyperphosphorylated form (inactive) β-catenin, which is phosphorylated at these residues, by western blot in fracture callus lysates at day 9 post-injury.

Alcohol exposure resulted in a 48% decrease in the levels of hypophosphorylated, activated β-catenin in day 9 fracture callus lysates. This reduction in active β-catenin parallels the 53% decrease in total β-catenin levels previously reported at day 9 post-fracture (Lauing et al. 2012). Although not significant, LiCl treatment increased active, hypophosphorylated β-catenin levels in the alcohol + LiCl group by 52% compared to the group treated with alcohol alone. Interestingly, LiCl treatment caused a significant decrease in levels of active β-catenin in the saline/LiCl group (**Figure 17)**.

Concurrently, we also observed that alcohol-exposed mice exhibited a significant increase in the amount of hyperphosphorylated β-catenin, suggesting that a greater proportion of cytosolic β-catenin in alcohol-exposed mice was targeted for degradation. Moreover, LiCl treatment decreased levels of hyperphosphorylated, inactive β-catenin levels in alcohol-exposed mice to levels observed in saline control mice (**Figure 18**).

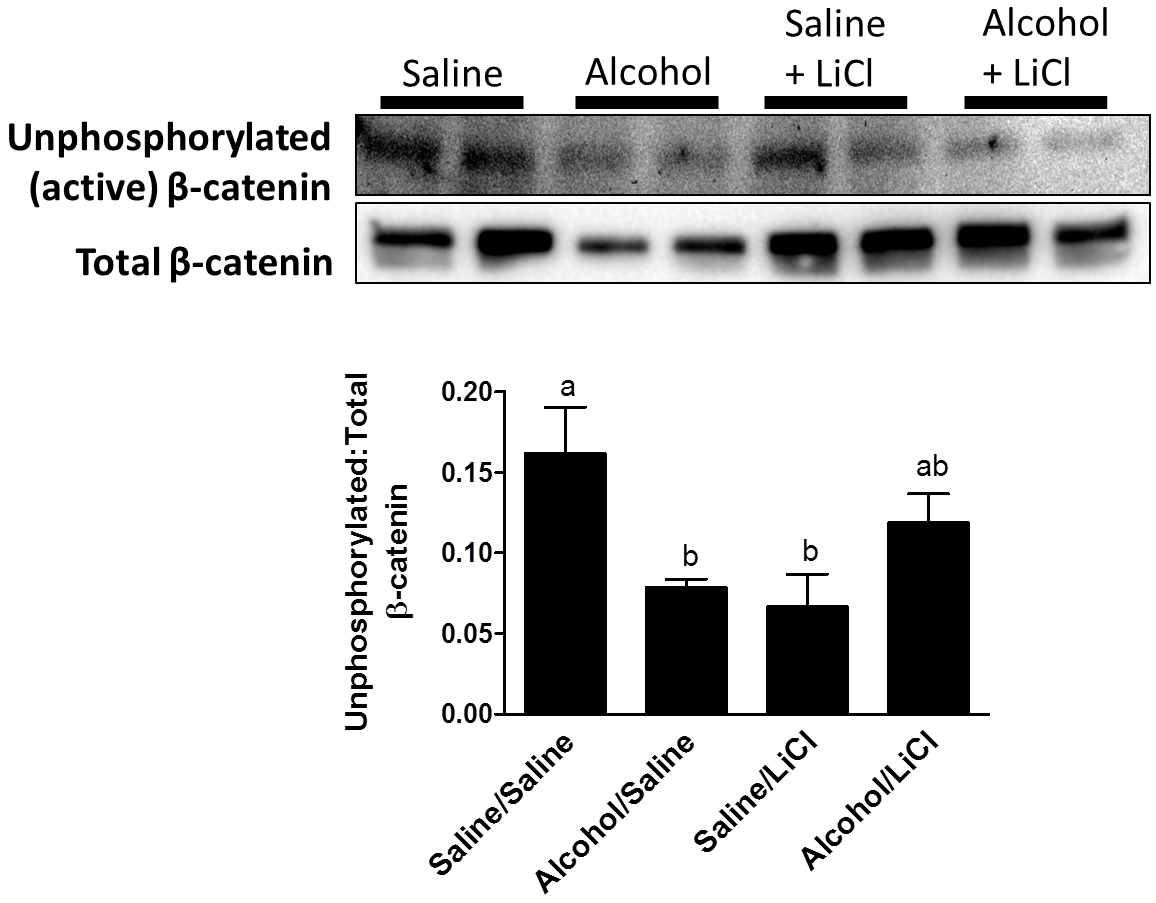

Figure 17: Effects of LiCl treatment on β-catenin activation following binge alcohol treatment. Representative western blots for activated and total β-catenin protein levels in fracture callus lysates at day 9 post-fracture. Data are presented as the densitometric ratio of activated/total β-catenin. Alcohol causes a significant decrease in the ratio of active (hypophosphorylated) β-catenin when compared to saline-exposed mice. LiCl treatment caused a 52% increase in activated β-catenin compared to alcohol treatment alone. Groups not sharing a letter are statistically significant, $p \leq 0.05$ using one-way ANOVA and Tukey's multiple comparison procedure. n=4-8 mice/treatment group.

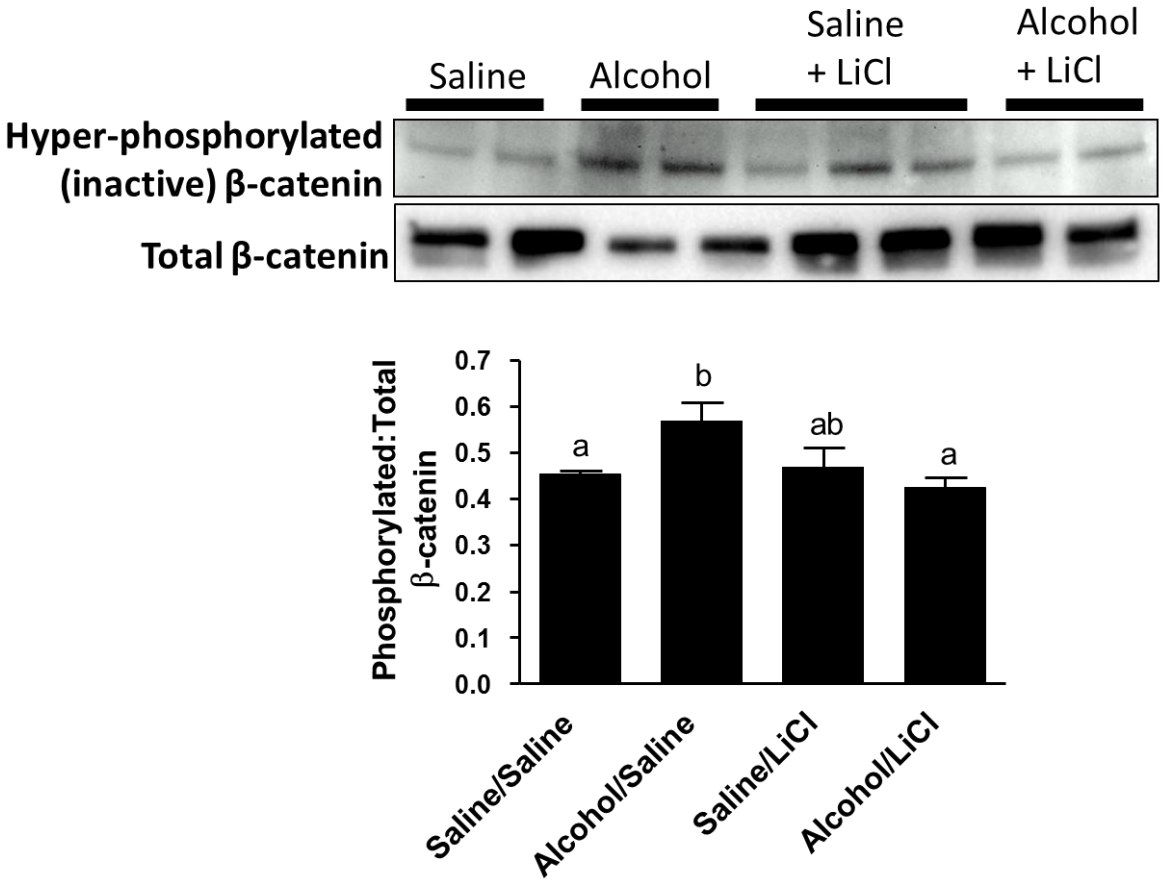

Figure 18: Effects of LiCl treatment on β-catenin hyperphosphorylation and inactivation following binge alcohol treatment. Representative western blots for hyperphosphorylated (inactivated) and total β-catenin protein levels in fracture callus lysates at day 9 post-fracture. Data are presented as the densitometric ratio of hyperphosphorylated/total β-catenin. Alcohol exposure resulted in a significant increase in the ratio of inactive β-catenin compared to saline-exposed mice. LiCl treatment significantly decreased hyperphosphorylated β-catenin levels to those found in saline-exposed mice. Groups not sharing a letter are significant, $p \leq 0.05$ using one-way ANOVA and Tukey's multiple comparison procedure. n = 4-8 mice/treatment group.

4. Effects of Acute Binge Alcohol Exposure and Lithium Chloride Treatment on Active and Inactive GSK-3β Protein Levels

Since GSK-3β is the principal negative regulator of canonical Wnt signaling and lithium potently inhibits this enzyme (Davies et al. 2000), we next investigated whether LiCl treatment significantly reduced hyperphosphorylated β-catenin levels in alcohol-treated mice by decreasing excessive GSK-3β activation. As shown in **Figure 19** (arrow), only the bottom band was quantified, since the antibody utilized cross reacts with a similar sequence containing a tyrosine phosphorylation site on the slightly heavier GSK-3α. In the alcohol-treated group, there is a significant increase in the ratio of activated, Tyr216 phosphorylated GSK-3β at day 9 post-fracture. These data correlate with the alcohol-induced decrease in activated, unphosphorylated β-catenin at day 9 post-fracture. LiCl treatment was able to completely restore levels of activated GSK-3β to the baseline levels observed in the saline-exposed control group. LiCl treatment did not result in further decreases in GSK-3β activation in the saline-exposed group.

In parallel experiments, alcohol-exposed mice demonstrate significantly decreased levels of deactivated, Ser9-phosphorylated GSK-3β in day 9 fracture callus tissue (**Figure 20**). LiCl treatment significantly increased the amount of deactivated GSK-3β in alcohol-exposed mice, revealing that the LiCl dose administered was sufficient to increase the proportion of Ser9-phosphorylated GSK-3β in the fracture callus to levels seen in saline control mice. No effect of LiCl treatment was noted on Ser9-phosphorylation of GSK-3β between the saline-exposed groups.

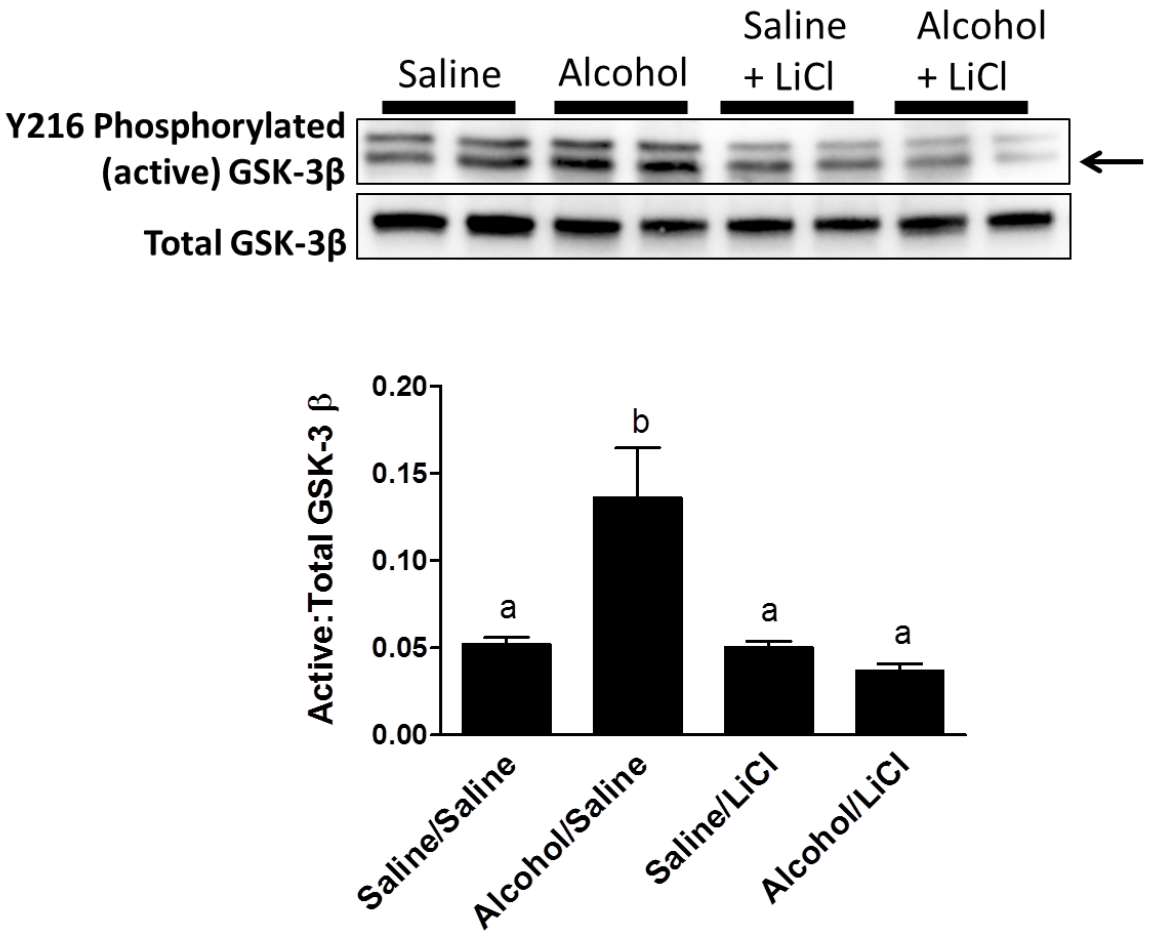

Figure 19: Effects of LiCl treatment on activated GSK-3β levels following alcohol exposure. Representative western blots for Tyr216-phosphorylated (activated) and total GSK-3β protein levels in fracture callus lysates at day 9 post-fracture. Data are presented as the densitometric ratio of activated/total GSK-3β. Only the bottom band (arrow) was quantified, as cross-reactivity with the heavier GSK-α also occurs with this antibody. Alcohol exposure resulted in a significant increase in the ratio of activated GSK-3β compared to saline-exposed mice. LiCl treatment significantly decreased activated GSK-3β to levels found in saline-exposed mice. LiCl treatment did not change GSK-3β in the saline-exposed group. Groups not sharing a letter are significant, $p \leq 0.05$ using one-way ANOVA and Tukey's multiple comparison procedure. n = 4-8 mice/treatment group.

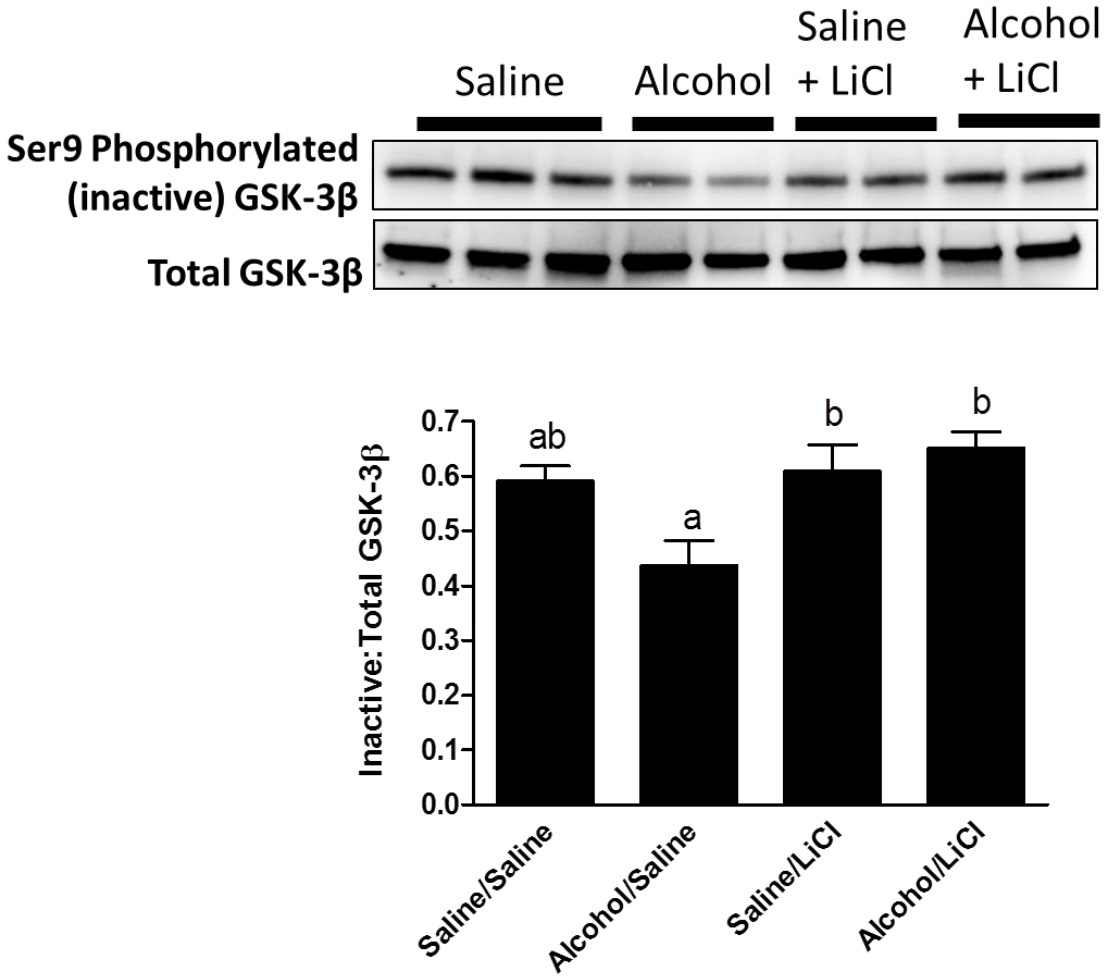

Figure 20: Effects of LiCl treatment on inactivated GSK-3β levels following alcohol exposure. Representative western blots for Ser9-phosphorylated (inactivated) and total GSK-3β protein levels in fracture callus lysates at day 9 post-fracture. Data are presented as the densitometric ratio of inactivated/total GSK-3β. Alcohol exposure resulted in a significant decrease in the ratio of inactivated GSK-3β compared to saline-treated mice, while LiCl treatment significantly increased the amount of inactivated GSK-3β to levels found in saline-exposed mice. Groups not sharing a letter are significant, $p \leq 0.05$ using one-way ANOVA and Tukey's multiple comparison procedure. n = 4-8 mice/treatment group.

DISCUSSION

We have previously demonstrated that during fracture repair in mice, acute binge alcohol exposure suppresses normal bone and cartilage formation, inhibits endochondral ossification, and decreases biomechanical strength of fracture calluses up to day 14 post-fracture. These alcohol-related effects are associated with perturbation of the levels of the canonical Wnt pathway protein β-catenin (Lauing et al. 2012). Experiments presented in this chapter confirm that a 2-week binge alcohol exposure not only decreases total β-catenin levels at day 9 post-fracture, but proportionately decreases the pool of hypophosphorylated, active β-catenin while increasing the amount of β-catenin targeted for degradation. These findings support our previous observations which demonstrate a decrease in β-catenin/TCF transcriptional activation in post-fracture day 9 callus tissue (Lauing et al 2012).

Deregulation of β-catenin during fracture repair has been shown to cause delayed healing (Chen et al. 2007) and inhibit endochondral ossification in the fracture callus (Huang et al. 2012), Strict regulation of Wnt/β-catenin signaling is also essential for normal cartilage formation and for the progression of chondrocytes to hypertrophy (Yang et al. 2012, Tamamura et al. 2005, Ryu et al. 2002). The findings presented in this chapter suggest that in alcohol-exposed mice, the absence of cartilage tissue and endochondral ossification is directly linked to deregulation of β-catenin levels, since LiCl treatment was able to restore both endochondral ossification and stabilized β-catenin levels following alcohol exposure. Chen et al. also showed that enhancement of the Wnt pathway using LiCl treatment must be precisely timed in order to promote fracture repair.

When administered several days following the injury, LiCl treatment enhanced mineralization of the fracture callus, however administration just prior to fracture resulted in the inhibition of fracture repair and the aggregation of undifferentiated tissue (Chen et al. 2007). These data also implicate the importance of maintaining the precise regulation of β-catenin levels during fracture repair. Collectively, these data support our hypothesis that the alcohol exposure deregulates the levels of stabilized β-catenin, which directly impacts the formation of mature cartilage and new bone in the fracture callus.

We hypothesized that fracture repair would be improved in alcohol-exposed mice by increasing stabilized β-catenin levels through suppression of increased GSK-3β activation with LiCl. Following LiCl treatment in alcohol-exposed mice, we observed a 52% increase in active β-catenin when compared to mice treated with binge alcohol alone, though this did not reach statistical significance. Similarly, we see an improvement in the biomechanical strength of alcohol-exposed mice following LiCl treatment, although this was not statistically different from the mice administered alcohol alone. *In vitro* experiments have shown that a 10-fold increase in unphosphorylated β-catenin following LiCl treatment increases TCF-dependent transcription by over 200-fold (Staal et al. 2002). These results suggest that a small increase in hypophosphorylated β-catenin, such as that seen in the alcohol-exposed mice receiving LiCl, is sufficient to cause major changes in β-catenin nuclear localization and Wnt target gene expression and may translate to the restoration of normal healing we observe histologically in the alcohol/LiCl-treated mice.

At day 9 post-fracture, we also observe a significant decrease in activated β-

catenin in mice treated with saline and LiCl, when compared to mice exposed to saline alone. Since LiCl did not decrease activated GSK-3β levels in the saline-exposed mice, we can only speculate that the decrease in hypophosphorylated β-catenin was compensatory to maintain cellular homeostasis in cells required for fracture repair. Histologically and biomechanically, we do not see any significant effects of LiCl administration in saline-exposed mice on normal healing at day 14 post-fracture, indicating that the molecular changes we observe did not relate to any phenotypic changes caused by LiCl administration.

Stabilized β-catenin can participate in other pathways important for bone homeostasis, such as the FoxO-mediated pathway, which is stimulated during conditions of oxidative stress (Ambrogini et al. 2010, Rached et al. 2010). Chronic alcohol exposure has been linked to an increase in markers of oxidative stress and reactive oxygen species (ROS) (Chen et al. 2008, 2011). In response to increases in free radicals, the FoxO transcription factors require β-catenin binding to stimulate transcription of ROS scavenger enzymes to stabilize the cell, and competes with the TCF transcription factors for a small pool of stabilized β-catenin (Hoogeboom et al. 2008, Almeida et al. 2007). This could be especially detrimental during the fracture repair process, when canonical Wnt/TCF-mediated transcription is highly upregulated to promote bone and cartilage formation. In addition, accumulation of ROS *in vitro* leads to suppressed canonical Wnt/TCF signaling through deregulation of β-catenin (Shin et al. 2004, Almeida et al. 2007). Our laboratory recently reported that alcohol-induced impaired healing *in vivo* is attenuated by treatment with an antioxidant, N-acetylcysteine (NAC), suggesting that

binge alcohol exposure causes an increase in systemic oxidative stress (Volkmer et al. 2011). Taken together, these data provide an explanation for how the fracture healing process in mice receiving alcohol and LiCl may be confounded by alcohol-induced oxidative stress. Moreover, these data may partially explain why we do not see a complete recovery in stabilized β-catenin levels in alcohol/LiCl-treated mice or a statistically significant improvement in fracture callus strength.

One of the main negative regulators of canonical Wnt/β-catenin signaling is GSK-3β, which continually targets β-catenin for degradation in the absence of Wnt stimulation through hyperphosphorylation of the S33/S37/T41 residues at the N-terminus (Ikeda et al. 1998). Deregulation of GSK-3β activation is associated with several pathological disease states, demonstrating the potential negative impact of the alcohol-induced increase we report in this investigation. We observe that alcohol exposure causes an increase in Tyr216-phosphorylated (activated) GSK-3β in fracture callus tissue at day 9 post-fracture, which also correlates with 1) a significant decrease in activated β-catenin, 2) a significant increase in hyperphosphorylated β-catenin targeted for degradation and 3) a significant decrease in deactivated Ser9-phosphorylated GSK-3β. To our knowledge, this is the first report of the effects of *in vivo* alcohol exposure on GSK-3β phosphorylation. *In vitro* studies have observed similar effects following alcohol exposure, in which suppressed nuclear β-catenin levels are seen with a concomitant increase in active GSK-3β (Chen et al 2010, Vangipuram and Lyman 2011). Our findings indicate that LiCl treatment offset the significant increase in activated GSK-3β found in alcohol-exposed mice and improved stabilized β-catenin levels while significantly decreasing

hyperphosphorylated β-catenin levels. These data support the hypothesis that LiCl is able to enhance β-catenin signaling though its predicted mechanism of GSK-3β inhibition.

Given our conclusions, we also acknowledge that there are limitations to the utilization of LiCl. Prior investigations have characterized the pharmacokinetics of lithium in rodents, indicating that no differences are seen in lithium plasma concentrations following intraperitoneal or subcutaneous injections, and that the LD50 in C57Bl/6 mice is a dose of approximately 820 mg/kg (Smith 1976, 1978). The dose administered in these studies (100 mg/kg) is well below that which would cause toxicity in mice. We did not observe outward signs of toxicity, such as ataxia and tremor, and the serum levels we obtained 3 hours post-injection also suggest that we did not administer toxic amounts of lithium. Although widely used and characterized as a potent GSK-3β inhibitor, lithium has the capability to weakly inhibit several other kinases, including casein kinase 2 (CK2) and members of the mitogen activated protein (MAP) kinase cascade. However, lithium ions have been shown to inhibit the activity of GSK-3β more potently than other cellular kinases, making it an ideal inhibitor of GSK-3β for *in vivo* use (Davies et al. 2000).

We have also taken into account that during fracture repair, kinases other than GSK-3β may play a role in β-catenin stabilization. Protein kinase A (PKA) has been reported to phosphorylate β-catenin at Ser552 and Ser675, resulting in augmented TCF/LEF transcriptional activation (Taurin et al. 2006). However, the PKA-mediated phosphorylation of β-catenin does not affect its stabilization or cellular localization. In addition, PKA phosphorylation at these sites does not prevent GSK-3β-mediated

phosphorylation or protection from degradation (Taurin et al. 2006). These data suggest that while phosphorylation of β-catenin by other kinases may play a role in enhancing its transcriptional activity, they do not prevent β-catenin from being targeted for degradation by GSK-3β. Therefore, in conjunction with our earlier observations, the alcohol-induced deregulation of both GSK-3β and β-catenin levels and subsequent recovery by LiCl treatment described in this study are convincing evidence that the canonical Wnt signaling pathway is targeted by alcohol exposure in bone tissue.

In conclusion, we report that binge alcohol exposure significantly modulates activated β-catenin and GSK-3β levels at day 9 post-fracture. This is associated with decreased biomechanical strength at day 14 post-fracture and alterations in fracture callus tissue composition. Treatment with LiCl in binge alcohol-exposed mice beginning at 4 days post-fracture completely restored endochondral ossification, partially restored the fracture callus strength and stabilized β-catenin levels, and significantly decreased activation of GSK-3β to levels seen in control mice. Considering our previous observations, these data suggest that canonical Wnt signaling is a target of binge alcohol exposure during fracture repair. Patients at risk for alcohol-induced deficient fracture healing may benefit from exogenous enhancement of the canonical Wnt pathway, such as LiCl treatment.

CHAPTER V

BINGE ALCOHOL EXPOSURE INDUCES EARLY DEREGULATION OF β-CATENIN SIGNALING AND DECREASED TARGET GENE EXPRESSION IN THE FRACTURE CALLUS

ABSTRACT

Introduction: Binge alcohol exposure is a risk factor for fracture-related complications and is associated with 25-40% of all orthopaedic trauma cases. Tight regulation of β-catenin levels through activated GSK-3β, the main negative regulator of the pathway, is essential in the formation of new bone and cartilage during fracture healing. Stabilization of β-catenin promotes transcription of Wnt target genes required for osteoblast and chondrocyte differentiation immediately following injury. Beginning at day 6 post-fracture, we have previously shown that binge alcohol treatment decreases bone and cartilage formation at the fracture site, deregulates β-catenin protein levels, and disrupts Wnt transcriptional activity. This study aims to determine whether binge alcohol deregulates the expression of key Wnt target genes and the regulation of GSK-3β in the fracture callus during the early stages of healing.

Methods: C57BL/6 mice were exposed to i.p. alcohol (2g/kg) or saline for 3 consecutive days. One hour after the third injection, mice received a stabilized tibial fracture and were sacrificed 1, 2, or 3 days post-injury. Fracture sites were isolated and assessed for canonical Wnt protein levels and target gene expression at the site of injury.

Results: Blood alcohol levels averaged 200 mg/dl at the time of injury. RT-PCR analysis revealed that alcohol-treated mice displayed significantly decreased expression of Wnt target genes important for osteoblast and chondrocyte lineage commitment and differentiation, such as Runx2, Sox9, Wnt10b, and β-catenin. Alcohol was associated with a decrease in expression of the endogenous inhibitors SOST, Dkk1, and Sfrp4 only at day 3 post-fracture. Protein analysis revealed that alcohol increased the level of activated GSK-3β (Y216 phosphorylation) compared to saline controls at days 1 and 3 post-fracture. The deregulation of GSK-3β activation was associated with deregulation of stabilized β-catenin protein levels at the fracture site.

Discussion: These data show that alcohol exposure during fracture disrupts the tight regulation of Wnt/β-catenin signaling during early stages of healing. This regulation during the initial stages of fracture repair is particularly important for mesenchymal stem cells that are actively upregulating canonical Wnt target genes during the differentiation process in order to repair the injured bone.

INTRODUCTION

Fracture repair can be simplified into 4 main phases: the inflammatory phase, soft callus formation, hard callus formation, and the remodeling phase (reviewed in Einhorn 1998). The previous 2 chapters have demonstrated alcohol-induced perturbation of key events during the reparative phases of healing, including impaired formation of the cartilaginous fracture callus and inhibition of endochondral bone formation. However,

the molecular events leading to the formation of the callus tissue are tightly regulated in the early phases of repair, and canonical Wnt signaling plays an essential role in initiating target gene transcription of factors involved in osteoblast and chondrocyte development. Therefore, it is reasonable to hypothesize that binge alcohol exposure may disrupt the early events of fracture repair involved in Wnt-mediated chondrocyte and osteoblast formation, and ultimately lead to impaired fracture callus formation. In this chapter, we investigate the effects of binge alcohol exposure on the inflammatory phase, which begins immediately following the injury and lasts up to 1 week in humans and approximately 2 days in the mouse tibial fracture model utilized here (Hiltunen et al. 1993).

During the initial stages of fracture repair, endothelial cells of the injured blood vessels release a host of cytokines and chemokines to attract inflammatory cells to the site of injury, which promotes the formation of a hematoma surrounding the fracture site. Hematoma formation and the recruitment of inflammatory cells are critical for the recruitment of local and distant mesenchymal stem cells (MSC) to the injury site, which creates an undifferentiated mass of cells termed granulation tissue. Within the granulation tissue, it is presumed that canonical Wnt signaling is tightly regulated to promote the differentiation of the MSC into bone and cartilage cells. Therefore, alcohol-related perturbations in Wnt signaling during the earliest phases in fracture repair may be responsible for the functional and cellular defects that we observed at later time points. Currently, there are no data to demonstrate that binge alcohol exposure disrupts total β-

catenin protein levels or that alcohol exposure modulates GSK-3β activity in bone tissue in the inflammatory phase of fracture repair.

The canonical Wnt pathway, through β-catenin/TCF dependent transcription, controls the expression of many genes required for osteoblast and chondrocyte development. Previous chapters describe modulation of total β-catenin levels and deregulation of GSK-3β activation following binge alcohol exposure at day 9 post-fracture. However, it is important to understand the role of binge alcohol exposure on the Wnt signaling pathway during the critical stages of MSC differentiation into bone and cartilage. To investigate whether these alcohol-related effects are downstream of early Wnt deregulation at the site of fracture, we investigated protein levels of β-catenin and GSK-3β and Wnt target gene expression in the first days following injury in the bone and granulation tissue immediately surrounding the injury site. We hypothesized that binge alcohol exposure would result in decreased β-catenin levels, increased GSK-3β activation, and subsequent decreased target gene expression.

RESULTS

1. Binge Alcohol Effects on β-catenin Protein Expression and GSK-3β Activation at Day 1 Post-Fracture

Due to the importance of β-catenin signaling during MSC lineage commitment to the osteo-chondroprogenitor lineage, we investigated the effects of binge alcohol on total β-catenin protein expression at the fracture site at days 1 and 3 post-fracture. Alcohol exposure resulted in an 11% increase in total β-catenin levels at day 1 post-fracture

(p=0.07) compared to saline-treated mice (**Figure 21A**). To investigate whether the modulation of β-catenin levels were due to a deregulation in GSK-3β activity, we quantified the ratio of active (Tyr216) and inactive (Ser9) GSK-3β levels compared to total levels. At day 1 post-fracture in the alcohol-treated group, there was a significant decrease in the amount of Ser9 phosphorylated, deactivated GSK-3β compared to saline-treated groups (**Figure 21B**), despite an alcohol-induced increase in total GSK-3β levels at this time point (**Figure 21D**). Alcohol exposure also resulted in an increase in Tyr216-phosphorylated GSK-3β at day 1post-fracture, however the significant increase in total GSK-3β levels abolishes this difference.(**Figure 21C**).

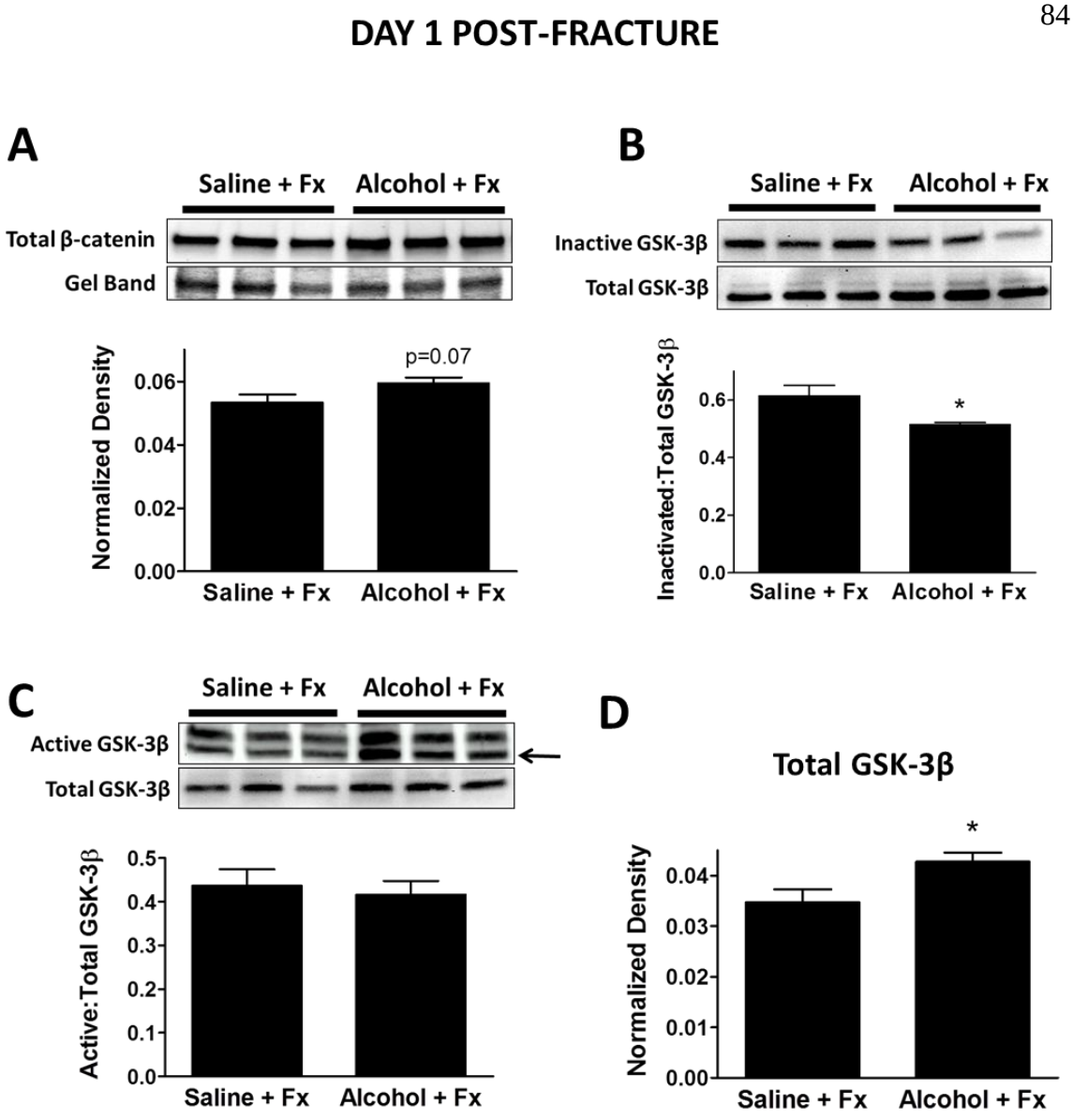

Figure 21. Binge alcohol effects on β-catenin protein expression and GSK-3β activation at day 1 post-fracture. Representative western blots for (A) total β-catenin, (B) inactivated Ser9-phosphorylated GSK-3β, (C) activated Tyr216-phosphorylated GSK-3β, and (D) total GSK-3β protein levels in the fracture site at day 1 post-fracture. Data are presented as the densitometric ratio of Coommassie-stained band density/total β-catenin or phosphorylated/total GSK-3β Alcohol exposure increased β-catenin ($p=0.07$) as compared to saline-exposed mice, which is accompanied by a significant decrease in inactivated GSK-3β. Alcohol increases activated GSK-3β, though this difference is abolished due to a significant increase in overall GSK-3β levels in the alcohol group. *$p<0.05$, Student's t test. n = 6 mice/treatment group.

2. Effects of Binge Alcohol on Active and Deactivated GSK-3β Levels During Early Fracture Repair

At day 3 post-fracture, alcohol-treated mice demonstrate a significant 34% decrease in total β-catenin protein levels (**Figure 22A**). This decrease is also associated with a significant increase in the amount of active GSK-3β compared to saline-treated mice (**Figure 22B**), which corresponds to the alcohol-related decrease in β-catenin levels at day 3 post-fracture. Alcohol did not cause a significant difference in the amount of inactivated GSK-3β present at the fracture site (**Figure 22B**), however alcohol exposure resulted in a significant increase in the amount of activated GSK-3β at day 3 post-fracture (**Figure 22C**). This increase in GSK-3β activation correlates with the significant decrease in β-catenin levels. Unlike day 1post-fracture, no significant modulation of total GSK-3β levels were noted at day 3 post-fracture (**Figure 22D**).

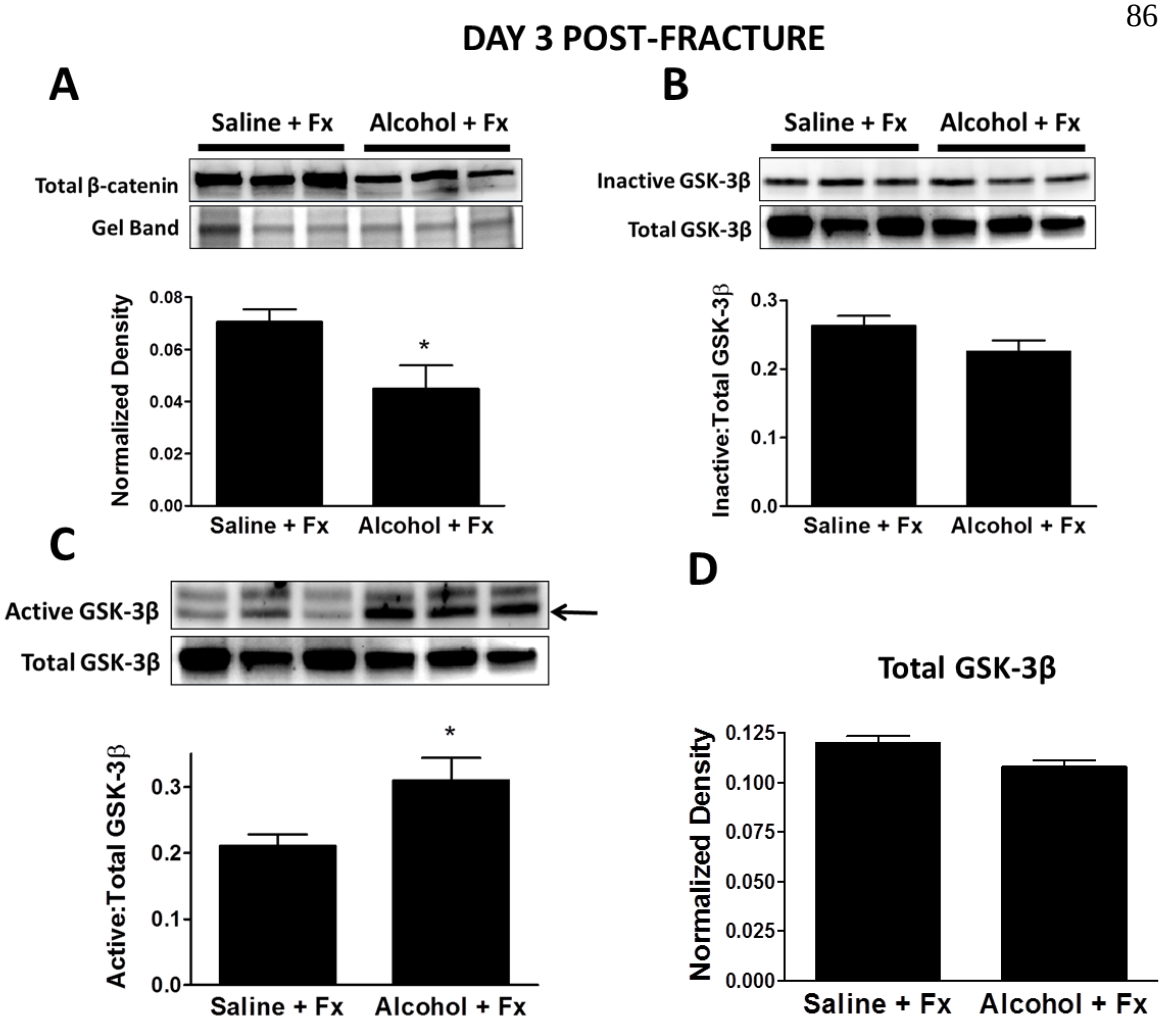

Figure 22. Binge alcohol effects on β-catenin protein expression and GSK-3β activation at day 3 post-fracture. Representative western blots for (A) total β-catenin, (B) inactivated Ser9-phosphorylated GSK-3β, (C) activated Tyr216-phosphorylated GSK-3β, and (D) total GSK-3β protein levels in the fracture site at day 3 post-fracture. Data are presented as the densitometric ratio of Coommassie-stained band density/total β-catenin or phosphorylated/total GSK-3β. Alcohol exposure significantly decreased β-catenin levels as compared to saline-exposed mice, which is accompanied by a significant increase in Tyr216 activated GSK-3β. Alcohol caused no measurable change in inactive Ser9 GSK-3β or total levels. *$p<0.05$, Student's t test. n = 8-9 mice/treatment group.

3. Effects of Binge Alcohol Exposure on Canonical Wnt Target Gene Expression at the Fracture Site

Due to the deregulation of β-catenin levels and GSK-3β activation states, we next wanted to determine whether this correlated with perturbation in the expression of canonical Wnt target genes at days 1-3 post-fracture. At day 1, we observe modest yet significant decreases in the mRNA expression of Wnt pathway genes, including β-catenin, GSK-3β, and APC. Wnt10b and Runx2, genes required for MSC commitment to the osteochondral lineage and osteoblast differentiation were also significantly decreased by alcohol exposure (**Figure 23**).

At day 2 post-fracture, there is a persistent alcohol-related decrease in the Wnt pathway genes β-catenin and GSK-3β. The classic Wnt target gene Axin is also decreased by alcohol exposure at day 2 post-fracture, along with the Wnt transcription factor Lef1. At this time point, alcohol exposure causes approximately a 50% reduction in the expression of both Runx2 and Sox9, which is an essential chondrocyte transcription factor. The Wnt target gene OPG, which inhibits osteoclast formation, is also significantly decreased. There are no alcohol-related changes in the expression of the endogenous Wnt inhibitors sclerostin (SOST), secreted frizzled-related protein 4 (Sfrp4), and Dickkopf-1 (Dkk1) at days 1 or 2 post-fracture (**Figure 24**).

At day 3 post-fracture, β-catenin and GSK-3β expression are still decreased following alcohol exposure. The chondrocyte transcription factor Sox9 remains significantly decreased and later-stage maturation markers of osteoblast differentiation, such as collagen 1 and osteocalcin (OCN) are now decreased in the alcohol group.

Interestingly, alcohol exposure was associated with a significant decrease in all 3 of the endogenous inhibitors that were measured - SOST, Sfrp4, and Dkk1 – at day 3 post-fracture.

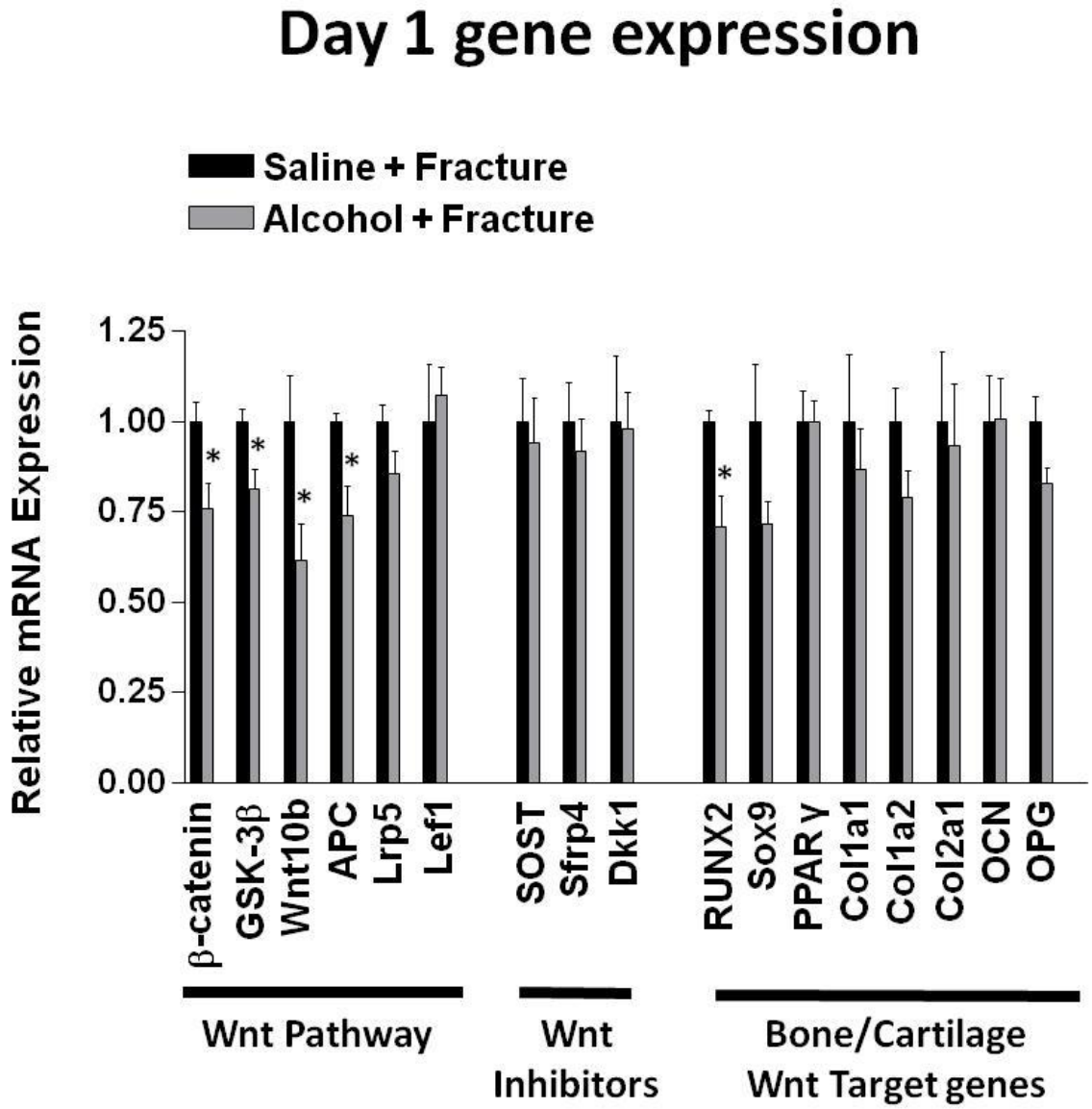

Figure 23: Effects of binge alcohol on canonical Wnt target gene expression at the fracture site day 1 post-injury. Relative mRNA levels at day 1 post-fracture were normalized to endogenous β-2 microglobulin gene expression and expressed as relative mRNA expression compared to saline-treated control mice. Genes highlighted in red demonstrate that alcohol exposure caused a significant decrease in mRNA expression, *$p<0.05$, n=5-6 mice/group.

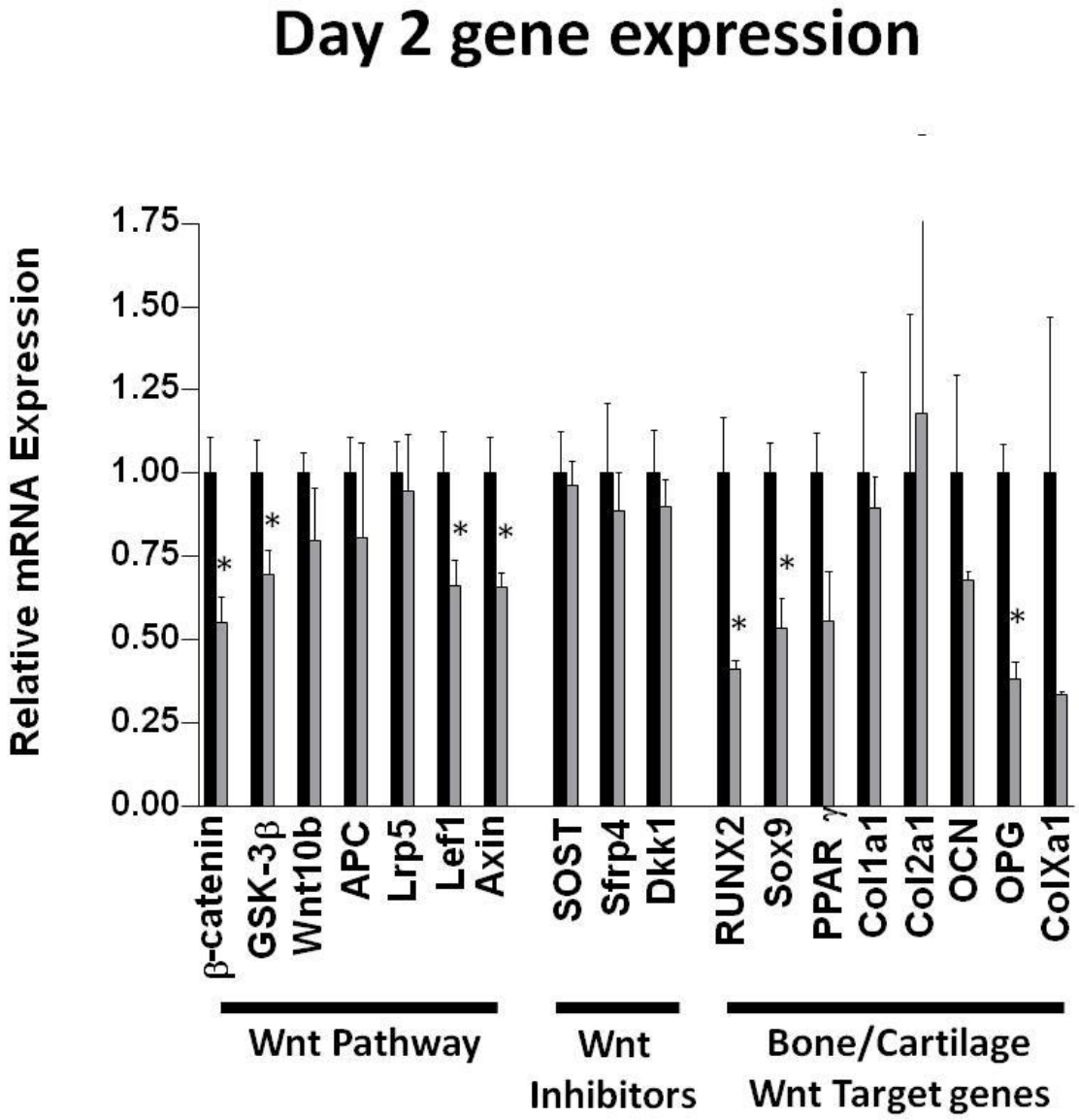

Figure 24. Effects of binge alcohol exposure on canonical Wnt target gene expression at the fracture site day 2 post-injury. Relative mRNA levels at day 2 post-fracture were normalized to endogenous β-2 microglobulin gene expression and expressed as relative mRNA expression compared to saline-treated control mice. Genes highlighted in red demonstrate that alcohol exposure caused a significant decrease in mRNA expression, *$p<0.05$, n=5-6 mice/group.

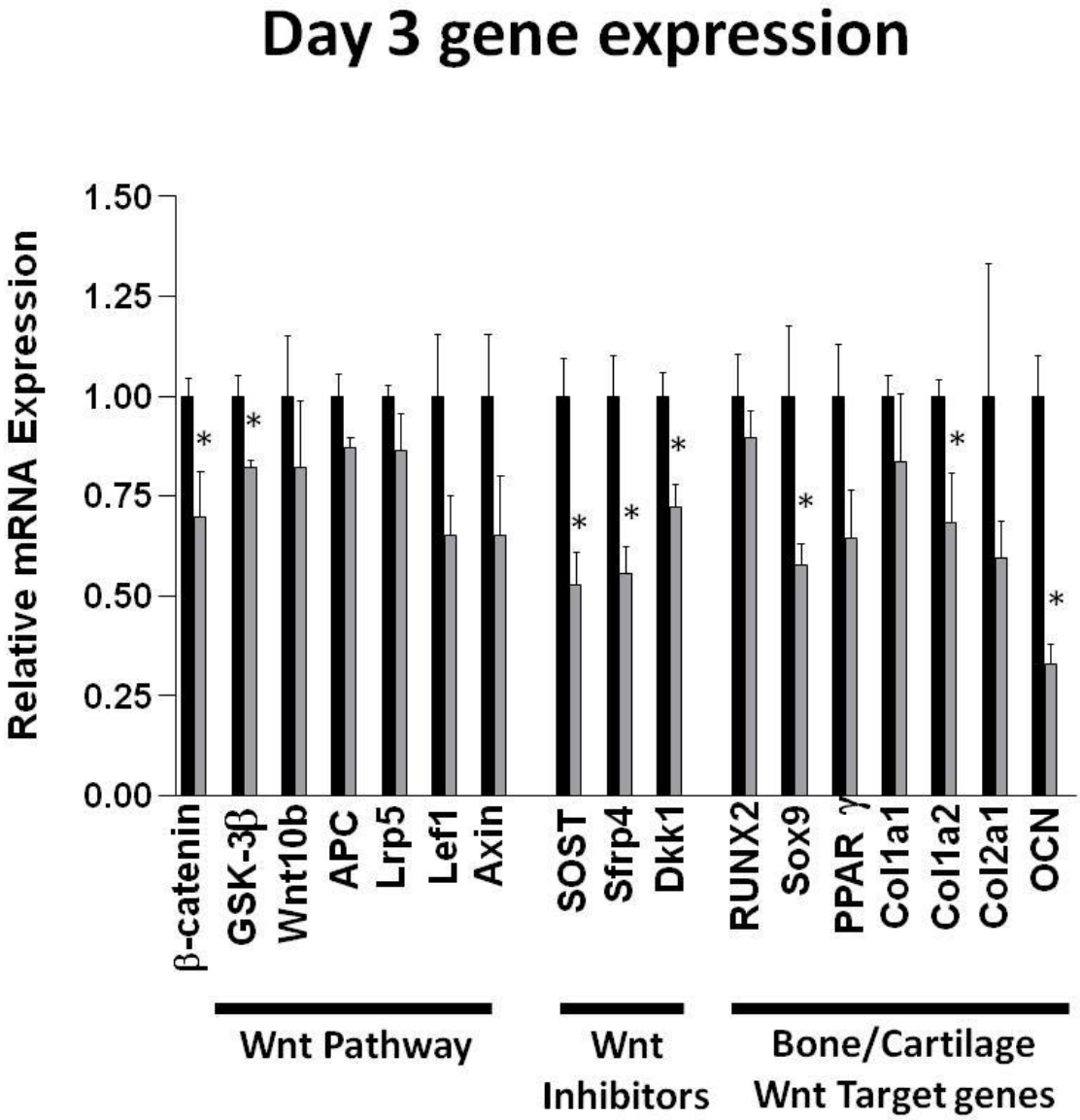

Figure 25. Effects of binge alcohol exposure on canonical Wnt target gene expression at the fracture site day 3 post-injury. Relative mRNA levels at day 3 post-fracture were normalized to endogenous β-2 microglobulin gene expression and expressed as relative mRNA expression compared to saline-treated control mice. Genes highlighted in red demonstrate that alcohol exposure caused a significant decrease in mRNA expression, *$p<0.05$, n=5-6 mice/group.

DISCUSSION

In this chapter, we provide evidence that binge alcohol exposure prior to fracture injury deregulates β-catenin and GSK-3β protein levels and suppresses canonical Wnt target genes important for osteoblast and chondrocyte differentiation at the fracture site. Though the data do not provide a complete explanation of the effects of alcohol exposure on Wnt signaling during the early healing phases, it provides some insight into how alcohol-induced modulation of β-catenin signaling may affect the later phases of fracture repair. Though β-catenin expression in the callus at days 1-3 is low compared to levels found in later stages of healing, quantifying hypo-phosphorylated and hyper-phoshorylated β-catenin levels would be useful to determine whether alcohol increases the amount of β-catenin targeted for degradation, as seen at day 9 post-fracture.

Since cartilage formation has not yet begun at the site of injury, the deregulation of mRNA levels we observe are likely within the granulation tissue that forms during the early phase of fracture repair, which contains undifferentiated MSC and osteo-chondroprogenitors. As described in previous chapters, the precise regulation of β-catenin is essential for fracture repair, MSC differentiation, and endochondral bone formation (Day et al. 2005, Chen et al. 2007, Huang et al. 2012). Though the changes reported here are modest, the small amount of tissue present at the injury site consists of undifferentiated MSC and progenitor cells, which are sensitive to molecular changes during the differentiation process. Therefore, small alterations in target gene expression and protein levels, such as those observed in the alcohol group, may lead to substantial changes in stem cell differentiation into bone and cartilage. The transcriptional changes

presented herein may partially explain why effects of alcohol exposure are seen through day 14 of healing, which is long after the last exposure.

In support of the argument that alcohol exposure may affect progenitor cell differentiation in the granulation tissue, we observe a significant decrease in the two essential transcription factors required for normal bone and cartilage development during early repair. Runx2 expression, which is directly controlled by Wnt/β-catenin signaling, is significantly decreased following alcohol exposure (Dong et al. 2006). Runx2 is an essential transcription factor that promotes osteoblast differentiation from MSC and is a target of canonical Wnt signaling (Ducy et al. 1997, Dong et al. 2006). A study in human fracture hematoma tissue indicated that Runx2 mRNA expression was upregulated between 6-72 hours in the granulation tissue, but little to no Runx2 expression was noted in the bone marrow (Kolar et al. 2011). This suggests that in humans, the majority of early Runx2 expression is likely derived from proliferating osteoblasts and osteoblast precursors in the periosteal callus during the earliest phases of fracture repair, which are the cells responsible for creating the "anchoring" or periosteal callus tissue. Alcohol exposure may play a role not only in the development of MSC, but also in existing osteoblasts residing in bone adjacent to the fracture site.

Furthermore, we observe a decrease at days 2 and 3 post-fracture in Sox9 expression. β-catenin directly associates with Sox9, which is an essential transcription factor needed for mesenchymal condensation and subsequent chondrocyte differentiation (Akiyama et al. 2002, 2004). Sox9 is also expressed in pools of mesenchymal precursors that ultimately give rise to osteo-chondroprogenitor cells (Akiyama et al. 2005). The

significant decrease in these two transcription factors in the early healing phases provides evidence that alcohol may directly impact the differentiation of bone and cartilage at the fracture site.

Our laboratory has previously shown that alcohol causes a global upregulation of genes following binge exposure (Himes et al. 2008). An exception to this pattern was the identification of the canonical Wnt pathway, in which the majority of the pathway genes modulated by alcohol were significantly reduced. The data presented here confirm these observations, as seen by an overall decrease in canonical Wnt target gene expression, indicating that the suppression of the Wnt pathway by alcohol is a targeted effect and alcohol does not cause a global genome-wide decrease in expression. Interestingly, we observe a significant increase in the mRNA expression of endogenous inhibitors of the canonical Wnt pathway at day 3 post-fracture. This occurs concomitantly with a decrease in total β-catenin protein levels as well as a decrease in β-catenin mRNA expression. Conversely, alcohol causes an increase in GSK-3β activation despite a decrease in mRNA expression. GSK-3β and β-catenin are highly regulated by post-translational modifications. Therefore, the alcohol-related decrease in the mRNA expression of the Wnt target genes tested, including β-catenin and GSK-3β, is indicative that alcohol suppresses canonical Wnt activation and subsequently results in decreased target gene expression.

Several investigations have focused on gene expression profiling in rodent bone tissue after injury, and report that fracture repair is extremely complex and involves the upregulation of thousands of genes (Hadjiargyrou et al. 2002, Rundle et al. 2005),

including robust upregulation of Wnt pathway genes. Due to the complexity of the pathway, these data can only provide a modest interpretation of the early effects of alcohol exposure on the Wnt pathway during bone repair. However, given our conclusions from the previous data, the small changes observed may be sufficient to initiate a cascade of events that ultimately lead to decreased biomechanical strength, decreased bone and cartilage at the fracture site, and persistent modulation of β-catenin protein levels in the fracture callus.

CHAPTER VI

SUMMARY AND DISCUSSION

The deleterious effects of binge alcohol exposure prior to fracture injury in the murine model established in these experiments is of clinical relevance and is the first to report that alcohol specifically targets Wnt/β-catenin signaling during healing. Binge alcohol-treated mice display early deregulation of GSK-3β, the main negative regulator of the canonical Wnt pathway, modulation of total β-catenin levels, and decreased Wnt target gene expression at the fracture site beginning at post-fracture day 1. The impact of binge alcohol prior to injury persists through the intermediate phases of fracture repair, in which cartilage and bone appear in the callus tissue. At days 6 through 14 post-fracture in the alcohol group, there is continued disruption of total and stabilized β-catenin levels, which correlates with the alcohol-associated disruption in the amount of β-catenin/TCF-dependent transcriptional activation in the callus tissue at these time points. Given the significance of Wnt/β-catenin signaling during cartilage and bone differentiation in the fracture repair process, these alcohol-induced perturbations ultimately lead to the decrease in bone and cartilage present at the fracture site, and contribute to a significant reduction in fracture callus load-bearing capabilities. Furthermore, administration of LiCl treatments in the alcohol-treated mice to restrict GSK-3β-mediated phosphorylation

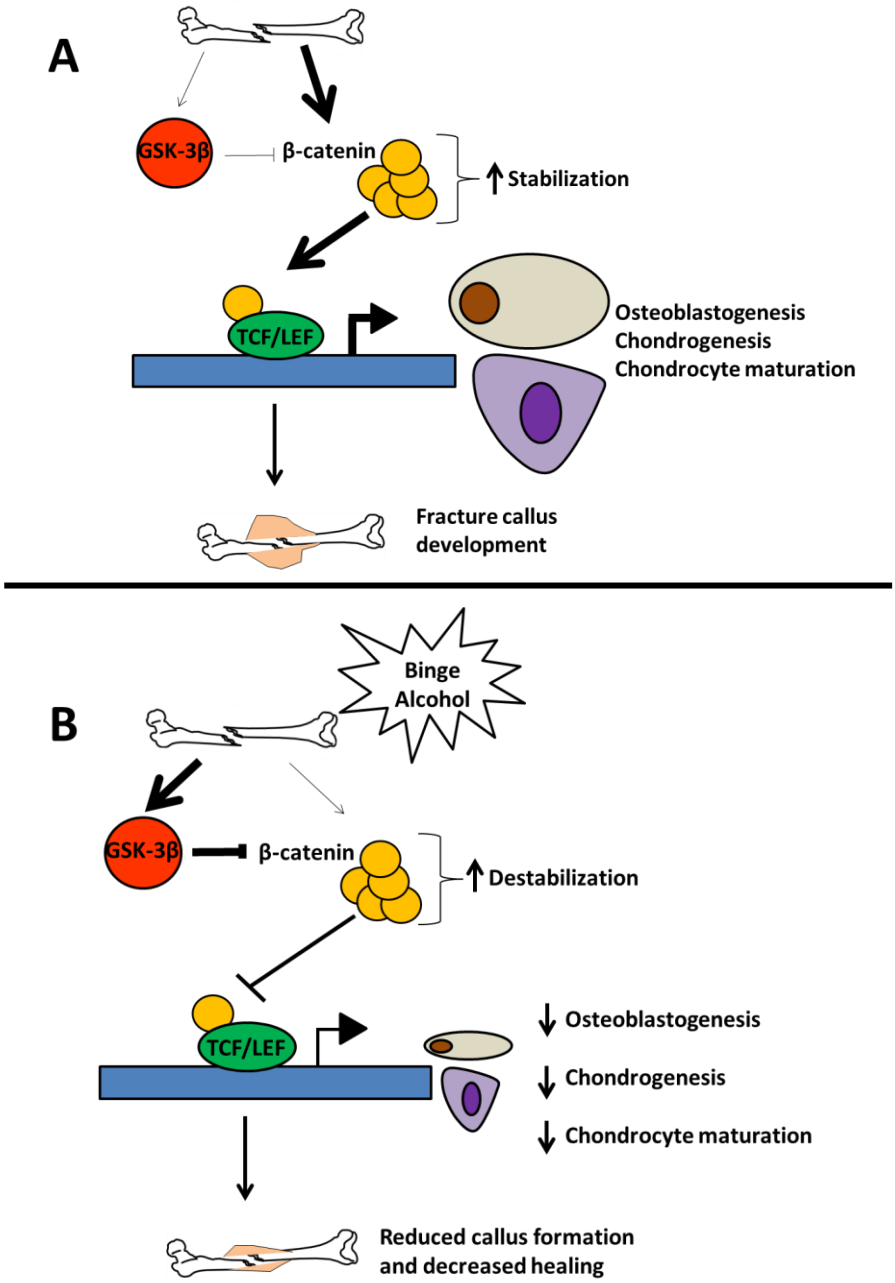

Figure 26. Proposed mechanism of binge alcohol-induced impaired fracture healing. A). Normal fracture repair causes stimulation of the canonical Wnt pathway and stabilization of β-catenin, leading to its nuclear translocation. The binding of β-catenin to TCF/LEF transcription factors leads to target gene expression required for bone and cartilage formation in the fracture callus. B). Binge alcohol exposure increases activation of GSK-3β, which deregulates the Wnt pathway and leads to destabilization of β-catenin. As a result, there is decreased β-catenin-driven TCF/LEF transcription and reduced cartilage and bone tissue at the fracture site, leading to impaired healing potential.

of β-catenin either improved or completely restored normal fracture healing parameters through decreased hyperphosphorylation of β-catenin. **Figure 26** proposes a potential scenario by which alcohol disrupts fracture repair and leads to decreased healing potential according to the data presented in the previous chapters.

We observe an incomplete recovery of fracture callus biomechanical strength and β-catenin levels following lithium chloride treatment, implicating the involvement of other pathways besides Wnt signaling during fracture repair. One possible explanation is that alcohol increases oxidative stress in bone cells (Chen et al. 2008, 2010),and it is established that the FoxO/β-catenin pathway is activated rapidly following exposure to oxidative stress in bone. The FoxO transcription factors have a higher affinity for β-catenin than the TCF transcription factors, which shifts the pool of β-catenin toward the FoxO pathway if oxidative stress is present (Hoogeboom et al. 2008, Ambrogini et al. 2010, Rached et al. 2010). Therefore, alcohol-induced oxidative stress may cause competition for the small pool of stabilized β-catenin even in the presence of lithium chloride, and prevent complete recovery of bone strength and β-catenin. Nonetheless, enhancement of the canonical Wnt pathway greatly improved fracture healing parameters in alcohol-treated mice, suggesting that the predominant pathway presiding over fracture healing is the Wnt/β-catenin pathway.

Clinically, these data may be useful for individuals that present with orthopaedic trauma and a history of alcohol abuse, since a greater proportion of these patients experience fracture healing complications such as delayed union. The identification of the Wnt pathway in these studies as a significant target of alcohol provides various

treatment targets in order to improve fracture healing in this population. Treatment with lithium chloride in this study significantly improved fracture repair in mice receiving alcohol by suppressing increased GSK-3β activity. Since lithium salts are currently in use clinically, this compound could easily be utilized as a treatment option for alcoholic patients at risk for nonunion, or patients that are lacking clinical signs of normal fracture repair.

APPENDIX A:

SPECIFIC METHODS

Acute Binge Alcohol Administration

Male C57Bl/6 mice 6-7 weeks of age were obtained from Harlan Laboratories (Indianapolis, IN) and housed in a facility approved by the Institutional Animal Care and Use Committee at Loyola University Medical Center. The mice were allowed to acclimate to the environment for 1 week prior to initiation of the experimental procedures. Animals were randomly assigned to either the Saline + Fracture group or the Alcohol + Fracture group. The acute alcohol exposure utilized in all experiments consisted of a single daily intraperitoneal injection of a 20% (v/v) ethanol/saline solution made from 100% molecular grade absolute ethanol (Sigma-Aldrich, St. Louis, MO) and sterile isotonic saline. Mice were administered the ethanol/saline solution at a dose of 2 g/kg once per day for 3 consecutive days, and were weighed daily prior to injection to ensure correct dosage. Mice in the saline control groups were administered sterile isotonic saline only. If mice were utilized for the biomechanical or LiCl studies in Chapter IV, the binge alcohol paradigm describe above was repeated for 2 weeks. One hour after the final injection, mice were subjected to the stabilized tibial fracture surgery described below. Blood alcohol levels averaged approximately 200 mg/dl at the time of fracture (1 hour post-injection)

Stabilized Tibial Fracture Surgery

1. Administer final i.p. injection of alcohol or saline.
2. Wait 25 minutes, and administer buprenex (0.1 mg/kg) s.c.
3. Wait 20 more minutes, and inject mice i.p. with an induction dose of anesthesia (0.5-0.75 mg/kg ketamine and 0.06-0.08 mg/kg xylazine). When mice appear sedated, remove hair from left hind limb using sterile cotton swabs in the surgical prep area. Administer 5 mg/kg s.c. prophylactic gentamicin.
4. Place mouse in sterile surgical area and place the nose into the isofluorane cone for complete anesthetization. Swab surgery site with povidone-iodine solution followed by 70% ethanol.
5. Create a small incision with sterilized small surgical scissors to expose the patellar tendon and extend the incision distally to expose the proximal third of the tibia.
6. Find the most proximal aspect of the tibia behind the patellar tendon and use a 27-gauge needle to ream a hole into the medullary cavity.
7. Remove the 27-gauge needle and insert a .25 mm-diameter insect pin (Fine Science Tools, Inc., Foster City, CA) into the reamed hole to stabilize the entire length of the tibia.
8. Cut the insect pin completely flush with the proximal tibia using small sterilized wire cutters. Use angled bone scissors (Fine Science Tools) to surgically create a mid-diaphyseal tibial fracture.
9. Close wound with 6-0 prolene sutures and irrigate with sterile saline. Remove mice from anesthesia and administer 1 mL of warmed isotonic saline s.c.
10. Place mice in clean cages on heating pads with free access to food and water. All mice receive 3 doses of post-operative buprenex subcutaneously (0.05 mg/kg) for pain control every 10-12 hours.
11. Mice that receive lithium chloride treatment are administered daily subcutaneous injections of 100 mg/kg LiCl (Sigma) dissolved in sterile saline at 5:00 pm.

Manual Bone Processing, Embedding, and Sectioning

1. Plate freshly harvested fractured or contralateral tibias in labeled tissue cassettes.
2. Fix in 10% neutral buffered formalin for at least 48 hours.
3. Decalcify in daily changes of 10% EDTA or Cal-Ex Decalcifier (Fisher Scientific) with gentle agitation for 5-7 days.
4. Rinse bones under running tap water for at least 15 minutes to wash off excess decalcification fluid. Dehydrate the tissue in series of histological-grade reagent alcohol (Fisher Scientific, Waltham, MA) solutions for 1 hour each in airtight pyrex containers:

   70% alcohol ---------- 1 hour
   80% alcohol ---------- 1 hour
   95% alcohol ---------- 1 hour
   95% alcohol ---------- 1 hour
   100% alcohol --------- 1 hour
   100% alcohol --------- 1 hour
   xylene ----------1 hour
   xylene ---------- 1 hour

5. Drain excess xylene and place cassettes into melted paraffin in a 60°C oven to infiltrate overnight.
6. After paraffin infiltration, partially fill a metal mold with melted paraffin. Remove bone from cassette and oriented into the mold at a 45° angle. Fit an embedding ring over the mold and fill completely with melted paraffin. Place molds on a cold plate or ice until paraffin is hardened.
7. Using a microtome, cut 5 µm sections from the paraffin blocks until mid-callus sections are identified (the space where the pin was is visible). Place desired sections onto charged slides (Fisher Scientific). Bake slides for at least 1 hour on a 60°C slide warmer to melt paraffin.

Hematoxylin and Eosin (H & E) Staining

Process the slides to be stained with the following steps:

1. Deparaffinize in 2 changes of xylene, 10 minutes each.
2. 2 x 100% alcohol, 20 dips each.
3. 2 x 95% alcohol, 20 dips each
4. 80% alcohol, 20 dips
5. 50% alcohol, 5 mins
6. Tap water, 20 dips
7. Harris hematoxylin, 8 minutes
8. Running tap water until clear
9. 0.5% acid alcohol (1 mL HCl in 200 mls 80% alcohol), 3 dips
10. Running tap water until clear
11. Ammonium hydroxide (6 drops in 200 mLs tap water), 10 dips
12. Running tap water, 3 minutes
13. 95% alcohol, 10 dips
14. Eosin Y, 5 dips
15. 3 x 95% alcohol, 20 dips each
16. 2 x 100% alcohol, 20 dips each
17. 2 x xylene, 5 minutes each
18. Coverslip with Permount (Fisher Scientific)

Four-point Biomechanical Testing

1. Harvest injured or contralateral tibias (as the uninjured control) 14 days post-fracture and store in formalin until experiment is to be performed.
2. Warm up Instron materials testing machine (Instron Corporation, Model 5544, Canton, MA) for 15 minutes. Set program to begin a new 4-point bending test. Set the test run speed to 0.5 mm/second. The end of the test should be set to occur when a 20% drop in peak load has occurred.
3. Place the lower loading cell on the block and ensure that it is not touching the upper cell. Run a test to make sure there is no friction between the two pieces. If the loading curve appears to remain at or around zero, you can proceed with testing. Otherwise, keep adjusting the bottom load cell until there is zero friction.
4. Raise the upper loading cell using the machine controls so you can load the tibia onto the lower cell. Using forceps, remove the tibia from formalin and gently place it perpendicular to the bars of the lower loading cell. Be sure that the callus tissue is centered between the bars.
5. Use the machine controls to lower the upper loading cell until it is as close to the tibia as possible without touching it. Begin the test.
6. Load-deflection curves will appear on the screen. After failure of the bone has occurred, the maximum load in Newtons (N) should be recorded for each sample.

Generating Bone Lysate for Isolation of Total RNA or Protein

1. Harvest injured or contralateral bones into 1.5 mL microcentrifuge tubes. Place tubes into liquid nitrogen to snap-freeze the tissue.
2. Keep tibias frozen at -80°C until ready to isolate protein, and always keep the tissue on dry ice during the isolation procedure.
3. Prepare the freezer mill by filling with liquid nitrogen and allowing to cool. Create a "prep" station by assembling the pulverizing tubes and keeping them immersed in liquid nitrogen.
4. Using a Dremel rotary tool (Dremel Inc., Racine, WI), trim the fracture callus tissue free of the surrounding bone on dry ice. For contralateral tibias, utilize the entire bone to obtain enough sample. Weigh the fragment of bone and record.
3. Using sterile forceps, transfer the bone fragment into the cooled pulverizing tube and add 1 mL of lysis buffer (RIPA buffer, protease inhibitor, phosphatase inhibitor cocktail) for protein isolation or 1 mL of TRIzol reagent (Ambion, Life Technologies) for RNA isolation to the tube.
4. Crush the bone fragment in a freezer mill (SPEX CertiPrep Inc., Metuchen, NJ) on the appropriate setting.
5. Collect the pulverized matter from the tube into a labeled, RNAse-free 50mL tube. Keep all pulverized samples frozen at -80°C until ready to isolate protein or RNA (see protocols below).

Total Protein Isolation:

1. Thaw samples on ice for 4-5 hours.
2. Pipet samples into labeled 1.5 mL microcentrifuge tubes. Pipet up and down at least 10 times to agitate the sample. Keep all samples on ice.
3. Vigorously vortex each sample for 15 seconds each.
4. Centrifuge for 20 minutes at 12,000 rpm at 4°C.
5. Transfer supernatant to a separate labeled 1.5 mL microcentrifuge tube on ice.
6. Perform BCA assay to determine total protein concentration and aliquot samples.

Total RNA Isolation:

1. Allow pulverized bone in TRIzol reagent to thaw at room temperature and transfer liquefied samples to a RNAse-free 15 mL tube.
2. Add 200 µl chloroform to each tube and vortex vigorously for 15 seconds each. Incubate at room temperature for 5 minutes.
3. Centrifuge samples at 10,000 rpm for 10 minutes at 4°C.
4. Pipet the aqueous layer into a fresh RNAse-free tube on ice.
5. Add 200 µl of 100% molecular grade ethanol to each sample and vortex.
6. Transfer the mixture to labeled filter/collection tube units and centrifuge at 12,000 x g for 30 seconds at room temperature. Discard filtrate and replace filter into same collection tube.
7. Wash filter membrane with 500 µl wash buffer (Ambion, Life Technologies), centrifuge for 30 seconds, and discard filtrate. Repeat 2 more times.
8. Elute RNA by adding 100 µl elution buffer (Ambion, Life Technologies) to the filter membrane in a fresh collection tube. Centrifuge for 30 seconds to get RNA. Use sample to immediately check RNA levels on the Nanodrop.

Western Blot Detection of Phosphorylated and Total Proteins

1. Load 25 ug of each sample into 4-20% TGX Ready-Gels (Bio-Rad Inc., Hercules, CA). Perform SDS-PAGE at 95 V for approximately 2.5 hours.
2. Transfer protein to PVDF membranes at 250 mA for 1 hour.
3. Rinse membrane in TBS, and block for 1 hour at room temperature in 5% milk-TBS-0.1% Tween-20.
4. If blotting for phospho-proteins, rinse the blot in TBS-Tween in 3 changes of TBS-Tween, 5 minutes each. If no phospho-antibodies will be used, skip this step.
5. If using phospho-antibodies, dilute antibody 1:1000 in 5% BSA-TBS-Tween. Otherwise, dilute primary 1:1000 in 1% milk-TBS-Tween. Incubate with gentle agitation overnight at 4°C.
6. Rinse blot in 5 changes of TBS-Tween for 5 minutes each.
7. Incubate in secondary antibody diluted 1:10,000 in 1% milk-TBS-Tween or 1% BSA-TBS-Tween (for phospho-antibodies) for 45 minutes at room temperature.
8. Rinse blot in 5 changes of TBS-Tween for 5 minutes each.
9. Incubate blot in chemiluminescent substrate solution (SuperSignal West Dura, Thermo Scientific).
10. Image blot on CCD camera and quantify band intensity using Bio-Rad Image Lab Software.
11. Rinse blot in TBS and strip of antibody using stripping buffer (Thermo Scientific). Incubate membrane in Coommassie blue stain for 1 hour at room temperature. Destain membrane for 5-10 minutes in 80% methanol/10% acetic acid.
12. Allow membrane to dry completely and image the Coommassie-stained bands as loading control.

X-Gal Staining for β-galactosidase in TCF Transgenic Mouse Bones

1. Harvest tibias from TCF-transgenic mice and place in 4% paraformaldehyde for 24 hours at 4°C.
2. Rinse bones 3 times with wash buffer (PBS, 0.02% NP-40, 1M $MgCl_2$).
3. Fix bones in 0.2% glutaraldehyde in PBS for 30 minutes at room temperature.
4. Rinse bones 3 more times in wash buffer.
5. Place bones in 15 ml polypropylene tubes containing staining 10 mls staining buffer

   Put tubes in a 37°C water bath and allow stain to penetrate for 48 hours.

   Staining buffer:

   PBS, pH 7.4
   1 mg/ml X-Gal (Fermentas, Inc., Glen Burnie, MD) dissolved in dimethylformamide
   5 mM potassium ferricyanide
   5 mM potassium ferrocyanide
   2 mM magnesium chloride
   0.01 % sodium deoxycholate
   0.02% NP-40

6. Remove bones from staining buffer and wash thoroughly with wash buffer.
7. Decalcify stained bones with daily changes of 10% EDTA pH 7.0 (will not harm stain) at 4°C for 5 days.
8. Process and embed tissues as described above. Counterstain with Neutral Red.

Real-Time RT-PCR

1. Convert 2 µg of total RNA into cDNA according to instructions for High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).
2. Dilute each cDNA sample to 4 ng/µl using nuclease-free water in a clear conical-bottom storage plate and store at 4°C or on ice while prepared PCR plate.
3. On each PCR plate, run each sample in duplicate or triplicate, and include wells for both the endogenous control (beta-2 microglobulin) and for desired target genes.
3. Pipet into each well of the PCR reaction plate 15 µl of reaction mixture:

   10 µl Master Mix (Applied Biosystems)
   4 µl nuclease-free water
   1 µl primer/probe (TaqMan)

4. Using a multichannel pipette, transfer 5 µl of each diluted cDNA sample from the storage plate into the appropriate wells of the PCR plate containing reaction mixture.
5. Seal plate with optical adhesive film and centrifuge plate for 30 seconds at 4°C.
6. Use the 7500 Fast System Software (Applied Biosystems) to set up the reaction parameters and begin reaction.
7. Normalize the relative expression of target genes tested to the endogenous expression of B2m (beta-2 microglobulin).

TABLE 1: ANTIBODIES

Antibody name	**Supplier/Product #**
Rabbit anti-mouse Total β-catenin Polyclonal	Upstate (Millipore) #06-734
Rabbit anti-mouse Non-phospho-β-catenin Ser 33/37/Thr41 Polyclonal	Cell Signaling #4270
Rabbit anti-mouse Phospho-β-catenin Ser33/S37/Thr41 Polyclonal	Cell Signaling #9561
Rabbit anti-mouse GSK-3β Total Polyclonal	Abcam ab97996
Rabbit anti-mouse GSK-3β Ser9 Polyclonal	Cell Signaling #9336
Rabbit anti-mouse GSK-3β Tyr216 Polyclonal	Abcam ab75745
Goat anti-rabbit IgG HRP-conjugated Polyclonal	Abcam ab6721

TABLE 2: PRIMER/PROBES

Gene symbol	Gene Name	Applied Biosystems TaqMan Assay ID
B2M	Beta-2 Microglobulin	Mm00437764_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1
APC	Adenomatous polyposis coli	Mm00545877_m1
Axin1	Axin1	Mm01299066_m1
Bglap-r	Osteocalcin	Mm03413826_mH
Col1a1	Collagen, type I, alpha 1	Mm00801666_g1
Col1a2	Collagen, type 2, alpha 2	Mm01165167_m1
Col2a1	Collagen, type 2, alpha 1	Mm01309562_g1
Ctnnb1	Catenin (cadherin associated protein), beta 1	Mm01350394_m1
Dkk1	Dickkopf-1	Mm00438422_m1
GSK3b	Glycogen synthase kinase 3 beta	Mm00444911_m1
Lef1	Lymphoid enhancer factor 1	Mm01310389_m1
Lrp5	Low density lipoprotein receptor-related protein 5	Mm01227476_m1
Pparg	Peroxisome proliferator activated receptor gamma	Mm01184323_m1
RUNX2	Runt-related transcription factor 2	Mm00501582_m1
Sfrp4	Secreted frizzled-related protein 4	Mm00840104_m1
Sost	Sclerostin	Mm00470479_m1
Sox9	Sex-determining regioin Y-box 9	Mm00448840_m1
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (OPG)	Mm01205928_m1
Wnt10b	Wingless related MMTV integration site 10b	Mm00442104_m1

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VITA

Kristen L. Lauing was born on November 7, 1981 in Lake Forest, Illinois to Rosemary and Ronald Lauing. Kristen received her secondary education at Warren Township High School in Gurnee, Illinois. In August of 2000, Kristen entered the University of South Carolina, majoring in Biology and minoring in Spanish. During her college education Kristen worked in the laboratory of Alan Waldman, Ph.D. investigating mammalian homologous recombination and DNA mismatch repair. Upon graduation, Kristen spent 2 years working in a cancer registry in Columbia, SC where she conducted clinical cancer research and data collection with the Palmetto Health Organization.

In August of 2006, Kristen was accepted to the Interdisciplinary Program at Loyola University Medical Center. In January of 2007, she decided to continue working under the mentorship of Dr. John J. Callaci examining the impact of acute binge alcohol exposure on the Wnt signaling pathway during fracture repair. From 2008-2010, Kristen received a pre-doctoral fellowship award from the Alcohol Research Program training grant, which is under the directorship of Dr. Elizabeth Kovacs. From 2010-2012 she was awarded a pre-doctoral Ruth L. Kirchstein National Research Service Award fellowship from the National Institutes on Alcohol Abuse and Alcoholism. From 2008-2010 Kristen served on the Graduate Student Council as co-president and as a student representative for the Cell Biology, Neurobiology and Anatomy program. She also received first place

in the oral competition of Loyola's St. Albert's Day of Research in 2011. Kristen will be pursuing a post-doctoral position at the University of Chicago, where she will be investigating the specific roles of novel enzymes and proteins involved in cartilage formation and maturation using murine models of chondrodysplasia.